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REVIEW PAPER

Photosynthetic complex stoichiometry dynamics in higher plants: biogenesis, function, and turnover of ATP synthase and the cytochrome b_6f complex

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Abstract

During plant development and in response to fluctuating environmental conditions, large changes in leaf assimilation capacity and in the metabolic consumption of ATP and NADPH produced by the photosynthetic apparatus can occur. To minimize cytotoxic side reactions, such as the production of reactive oxygen species, photosynthetic electron transport needs to be adjusted to the metabolic demand. The cytochrome b_6f complex and chloroplast ATP synthase form the predominant sites of photosynthetic flux control. Accordingly, both respond strongly to changing environmental conditions and metabolic states. Usually, their contents are strictly co-regulated. Thereby, the capacity for proton influx into the lumen, which is controlled by electron flux through the cytochrome b_6f complex, is balanced with proton efflux through ATP synthase, which drives ATP synthesis. We discuss the environmental, systemic, and metabolic signals triggering the stoichiometry adjustments of ATP synthase and the cytochrome b_6f complex. The contribution of transcriptional and post-transcriptional regulation of subunit synthesis, and the importance of auxiliary proteins required for complex assembly in achieving the stoichiometry adjustments is described. Finally, current knowledge on the stability and turnover of both complexes is summarized.

Key words: Assembly, assimilation, auxiliary protein, chloroplast ATP synthase, complex turnover, cytochrome b_6f complex, gene expression, photosynthetic electron transport, translation.

Photosynthetic complex stoichiometry adjustments during leaf development and environmental acclimation

Plants need to balance the photosynthetic production of ATP and NADPH with their metabolic consumption by the Calvin–Benson cycle and the subsequent reactions of primary metabolism. Otherwise, an excess electron transport capacity could result in a disturbance of the cellular redox poise and electron transfer to molecular oxygen. The production of different types of reactive oxygen species (ROS) at both photosystem II (PSII) and photosystem I (PSI) could result in oxidative damage to the photosynthetic apparatus itself, and might even activate cell death pathways via retrograde signals from the chloroplast to the nucleus (Danon *et al.*, 2006; Kim *et al.*, 2012).

Changes in environmental parameters can affect both photosynthetic electron transport rates and Calvin–Benson cycle activity. Fluctuations occurring on the time scale of seconds to hours mostly trigger rapidly reversible changes in photosynthetic light utilization and electron partitioning between $NADP^+$ reduction and alternative pathways (see below). However, the maximum light-saturated electron transport capacity of the photosynthetic apparatus remains unaltered.

Only long-term changes of environmental conditions and the developmental state of the plant trigger adjustments of the composition of the photosynthetic apparatus, which alter both the photosynthetic capacity to produce ATP and NADPH, and photosynthetic light utilization (see 'Long-term responses of the photosynthetic apparatus during leaf ontogenesis and environmental acclimation').

Plant responses to short-term imbalances between photosynthetic electron transport and the metabolic ATP and NADPH demand

When light intensities are non-saturating, photosynthesis is limited by the excitation rates of the photosystems, but not by the biophysical and biochemical processes of photosynthetic electron transport and the Calvin–Benson cycle. Because a significant fraction of the proton motive force (pmf) across the thylakoid membrane is stored as an electric field component ($\Delta\Psi$), under light-limited conditions the thylakoid lumen pH value usually is in a range of 7.5 to a minimum of 6.5 (Takizawa *et al.*, 2007). This is sufficient to drive ATP synthesis, but photoprotective mechanisms such as non-photochemical quenching (see below) are only weakly activated. Thus, a high quantum efficiency of photosynthetic CO_2 fixation is ensured (Fig. 1A). When the light intensity increases towards light saturation of photosynthesis, or when the Calvin–Benson cycle is slowed down, so that the metabolic consumption of ATP decreases, the availability of ADP and especially free orthophosphate (P_i) becomes limiting for ATP synthase (Fig. 1B). Rapid fluctuations in light intensity can also result in a major P_i limitation of ATP synthase: because the activation of downstream reactions converting the phosphorylated Calvin–Benson cycle intermediates into starch in the chloroplast and especially into sucrose in the cytosol is usually slower than changes in the Calvin–Benson cycle activity itself, phosphorylated intermediates of the Calvin–Benson cycle accumulate (Stitt and Grosse, 1988; Stitt and Schreiber, 1988). In all cases, the P_i limitation slows down proton efflux through the ATP synthase, and the thylakoid lumen becomes increasingly acidic. This activates multiple photoprotective processes as a feedback response to the imbalance between photosynthetic ATP production and its consumption (Sharkey and Vanderveer, 1989; Kanazawa and Kramer, 2002; Kiirats *et al.*, 2009). In Fig. 1B, the induction of photoprotective mechanisms is indicated by the red arrows.

Excess amounts of absorbed light can be harmlessly dissipated via rapidly reversible non-photochemical quenching of excitation energy in the PSII antenna bed (qE; indicated as red waves in Fig. 1B). qE is activated by the protonation of two glutamate residues in the PsbS protein, which facilitates the switch of the PSII antenna bed to a quenching conformation (Li *et al.*, 2004). Additionally, lumen acidification below a threshold value of ~6.8–6.5 activates the violaxanthin de-epoxidase (Takizawa *et al.*, 2007), which converts the accessory pigment violaxanthin into zeaxanthin (Fig. 1B). Zeaxanthin may stabilize the PSII antenna in its dissipative state, but the precise molecular mechanism is still a matter of debate (reviewed by Horton, 2012; Jahns and Holzwarth, 2012).

Besides inducing qE, the acidification of the thylakoid lumen can reversibly slow down linear electron flux by 'photosynthetic control' of plastoquinol re-oxidation at the cytochrome b_6f complex (Fig. 1B). Already at moderate lumen

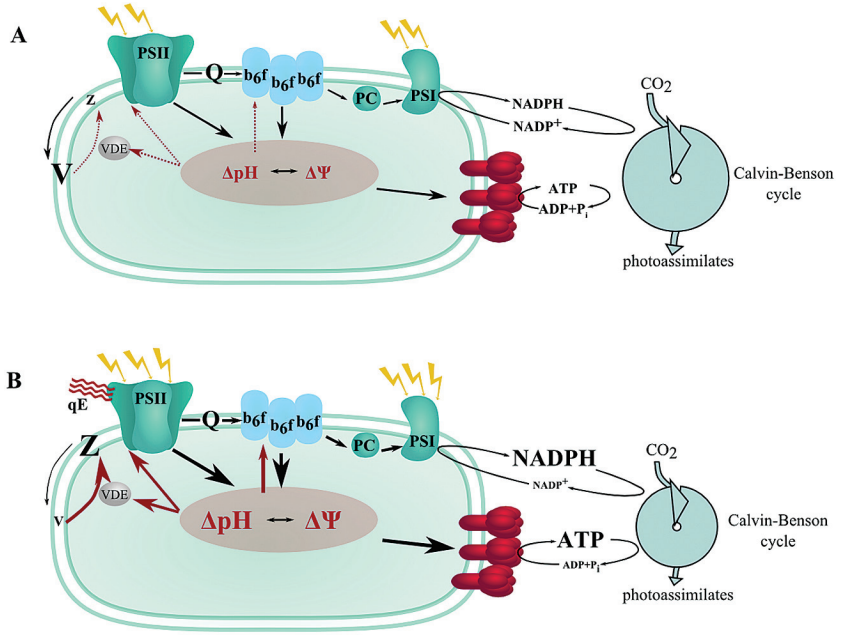

Fig. 1. Photoprotective mechanisms are controlled by the thylakoid lumen pH value. When photosynthesis is light limited, the pmf across the thylakoid membrane is in a range which allows efficient ATP synthesis to drive the Calvin–Benson cycle (indicated as a circle). However, photoprotective mechanisms such as qE are only weakly activated (A). When the actinic light intensity increases towards light saturation, or when the Calvin–Benson cycle is slowed down (indicated by the smaller size of the circle symbolizing the Calvin–Benson cycle), the metabolic consumption of ATP is decreased and ATP synthase becomes substrate limited by ADP and especially P_i (B). Then, the lumen rapidly acidifies to a level activating photoprotective mechanisms (indicated by red arrows) such as qE (symbolized by the red waves) and photosynthetic control of plastoquinol re-oxidation at the cytochrome b_6f complex. For details, please refer to the text.

pH values between 7.5 and 6.5, plastoquinol re-oxidation is the rate-limiting step of linear electron flux (Haehnel, 1984; Anderson, 1992; see the section below 'Cytochrome b_6f complex'). At lumen pH values <6.5, plastoquinol re-oxidation and proton release into the lumen become increasingly thermodynamically unfavourable (Takizawa *et al.*, 2007). Also, specific amino acid protonation events in the cytochrome b_6f complex contribute to this inhibition of plastoquinol re-oxidation (Nishio and Whitmarsh, 1993; Hope *et al.*, 1994; Kramer *et al.*, 1999; Takizawa *et al.*, 2007). In particular, photosynthetic control of linear electron flux protects the acceptor side of PSI against an over-reduction and a massive increase in the production of ROS.

This is most obvious in the *pgr5* mutant of *Arabidopsis thaliana*, which suffers from an impaired thylakoid lumen acidification and cannot properly induce photosynthetic control at the cytochrome b_6f complex. The molecular basis of the impaired lumen acidification in *pgr5* is still a matter of debate, but has been suggested to result either from a reduced cyclic electron flow (Nandha *et al.*, 2007; Joliot and Johnson, 2011) or from an impaired metabolic feedback inhibition and deregulation of ATP synthase activity (Avenson *et al.*, 2005). While the *pgr5* mutant only shows a moderate phenotype when grown under constant light conditions, under fluctuating light conditions, due to the impaired activation of photosynthetic control, PSI is highly susceptible to irreversible photoinhibition during the high-light pulses (Suorsa *et al.*, 2012). PSI photoinhibition results from the production of relatively long-lived ROS such as the superoxide anion and hydrogen peroxide (H_2O_2) on its acceptor side, which destroy the 4Fe–4S clusters on the PSI acceptor side (reviewed by Scheller and Haldrup, 2005). Additionally, ROS may diffuse from the PSI acceptor side and may damage other thylakoid components such as PSII (Krieger-Liszkay *et al.*, 2000).

In addition to qE and photosynthetic control, in the short term an imbalance between photosynthetic electron transport and metabolic ATP and NADPH consumption can be compensated by several flexibility mechanisms leading to a controlled reduction of alternative electron acceptors in the chloroplast (recently reviewed by Kramer and Evans, 2011; Schöttler and Tóth, 2014). There are two pathways which can transfer electrons originating from photosynthetic water oxidation at PSII back to molecular oxygen, thus again generating water. The Mehler–Asada cycle, also called the 'water–water cycle', detoxifies ROS produced at the PSI acceptor side, using ascorbate as the electron donor (reviewed by Asada, 1999). The plastid terminal oxidase (PTOX) can oxidize the plastoquinol pool under conditions when plastoquinone reduction by PSII exceeds the oxidation rate of the pool by the cytochrome b_6f complex, again resulting in the controlled reduction of molecular oxygen to water (Lennon *et al.*, 2003). Because water oxidation by PSII occurs on the luminal side, while oxygen reduction occurs on the stromal side of the thylakoid membrane, both reaction pathways contribute to generation of the pmf across the thylakoid membrane.

Excess reducing equivalents produced by the light reactions can be exported from the chloroplast via the malate shunt and other metabolic pathways (Scheibe, 2004). Finally, via the induction of cyclic electron flux or a down-regulation of ATP synthase activity, an increased thylakoid lumen acidification can trigger strong qE, and restrict linear electron flux by 'photosynthetic control' (Kohzuma *et al.*, 2009).

These flexibility mechanisms allow plants to minimize ROS production in response to a short-term imbalance between light reactions and the metabolic demand of primary metabolism. In the case of a long-term imbalance, the flexibility mechanisms may not be sufficient to avoid a harmful disturbance of the cellular redox poise and the accumulation of ROS. This is due to the relatively low capacity of each of these flexibility mechanisms in most higher plant species: the two water–water cycles can at maximum consume a few percent of light-saturated linear electron flux (Badger *et al.*, 2000; Driever and Baker, 2011; Shirao *et al.*, 2013). The overexpression of PTOX even seems to increase the level of oxidative stress when the plastoquinone pool is highly reduced, possibly due to H_2O_2 production as a side reaction of PTOX (Heyno *et al.*, 2009; Yu *et al.*, 2014). Also the capacity of the malate valve is limited (Backhausen *et al.*, 2000; Hebbelmann *et al.*, 2012). In the long term, a strong acidification of the thylakoid lumen, due either to the induction of cyclic electron flux or to a down-regulation of ATP synthase activity, is damaging to the photosynthetic apparatus (Krieger and Weis, 1993; Kramer *et al.*, 1999; Rott *et al.*, 2011). Therefore, in response to long-term changes in the metabolic demand, adjustments of the capacity of photosynthetic electron transport to produce ATP and NADPH via photosynthetic complex stoichiometry alterations are essential to ensure plant viability.

Long-term responses of the photosynthetic apparatus during leaf ontogenesis and environmental acclimation

Photosynthetic complex stoichiometry adjustments only occur in response to metabolic changes and environmental perturbations, which last at least for a few hours, and the complete acclimation response may require several days (reviewed by Walters, 2005; Schöttler and Tóth, 2014). Complex stoichiometry adjustments alter both the capacity of photosynthetic electron transport to produce ATP and NADPH and photosynthetic light utilization. Stoichiometry adjustments in response to a large number of environmental perturbations and during leaf development have been recently reviewed (Schöttler and Tóth, 2014). Here, we will only highlight a few selected examples.

During leaf ontogenesis, large changes in leaf assimilation capacity occur. Usually, leaf assimilation capacity peaks shortly before or as soon as the leaf is fully expanded. Then, drastic differences in the ontogenetic programme of the leaf occur between plant species. Dicotyledonous plants with strong sink organs maintain high assimilation capacities of all leaves for a long time, and undergo a relatively rapid loss of assimilation capacity only when the sink demand strongly decreases. Other dicots such as tobacco (*Nicotiana tabacum*), which have much smaller sink organs, start slowly but continuously to decrease the assimilation capacity of their leaves

even before the leaf is fully expanded. This decrease is attributable to the parallel down-regulation of the cytochrome b_6f complex, ATP synthase, and of the mobile redox carrier plastocyanin (Schöttler *et al.*, 2004, 2007). The contents of the two photosystems and their light-harvesting complexes (LHCs) remain largely unaltered until the final phases of leaf senescence. When photoassimilate export from source leaves is blocked due to the expression of an apoplastic invertase in tobacco, photosynthesis is already repressed in young leaves, and that again is largely due to a repression of Calvin–Benson cycle enzymes in parallel with the cytochrome b_6f complex, plastocyanin, and ATP synthase (von Schaewen *et al.*, 1990; Stitt *et al.*, 1991; Schöttler *et al.*, 2004).

Long-term drought stress restricts CO_2 availability to the Calvin–Benson cycle due to stomatal closure. It also directly affects the activities of some Calvin–Benson cycle enzymes (reviewed by Flexas *et al.*, 2004; Lawlor and Tezara, 2009). Both sunflower (*Helianthus annuus*) and wild watermelon (*Citrullus lanatus*) adjust their ATP and NADPH production by the down-regulation of chloroplast ATP synthase (Tezara *et al.*, 1999; Kohzuma *et al.*, 2009). In wild watermelon, the cytochrome b_6f complex content is also repressed (Kohzuma *et al.*, 2009). The contents of the two photosystems are not adjusted to a similar extent. The repression of ATP synthase decreases the rate of proton efflux from the thylakoid lumen and thereby strongly induces photoprotective qE and photosynthetic control of linear electron flux.

Large changes in complex stoichiometries also minimize ROS production in response to different growth light intensities. In dim light, low excitation rates per PSII reaction centre could result in a high lifetime of the plastosemiquinone radical on the PSII acceptor side and in charge recombination between Q_B^- and the S2/S3 states of the oxygen-evolving complex, ultimately resulting in ROS production in PSII (Keren and Krieger-Liszkay, 2011). In low-light-acclimated plants, ROS production and PSII photoinhibition may be avoided by an increased abundance of LHCII and a parallel repression of PSII reaction centres, which strongly increases the antenna cross-section per PSII. The contents of the cytochrome b_6f complex, ATP synthase, plastocyanin, and most Calvin–Benson cycle enzymes are low, because photosynthesis is limited by excitations rate, and not by the capacity of the biochemical reactions (reviewed by Schöttler and Tóth, 2014). With increasing growth light intensity, the limitation of photosynthesis shifts towards the biophysical and biochemical reactions of photosynthetic electron transport and the Calvin–Benson cycle. Accordingly, light intensity effects on the two photosystems are relatively moderate: on a chlorophyll basis, the amount of LHCII is reduced, while PSII reaction centre contents increase with growth light intensity, so that the total amount of chlorophyll associated with each PSII–LHCII unit remains constant. PSI contents are largely unaffected by growth light intensity (Leong and Anderson, 1984; Chow and Anderson, 1987; Chow and Hope, 1987; Chow *et al.*, 1988; De la Torre and Burkey, 1990; Burkey and Wells, 1996; Bailey *et al.*, 2001).

Instead, increased light intensities mostly affect the rate-limiting components of the light reactions and the Calvin–Benson cycle. Cytochrome b_6f complex contents increase with growth light intensity in a large number of species such as spinach (*Spinacia oleracia*; Chow and Hope, 1987), pea (*Pisum sativum*; Leong and Anderson, 1984; Chow and Anderson, 1987; Evans, 1987), *Alocasia macrorrhiza* (Chow *et al.*, 1988), barley (*Hordeum vulgare*; De la Torre and Burkey, 1990), soybean (*Glycine max*; Burkey and Wells, 1996), and tobacco (Yamori *et al.*, 2010; Schöttler and Toth, 2014). Changes in electron transport capacity and leaf assimilation closely correlate with cytochrome b_6f complex content (Evans, 1987; Chow *et al.*, 1988; Yamori *et al.*, 2010; Schöttler and Toth, 2014). Light intensity effects on ATP synthase have been less frequently studied, but in these studies ATP synthase activity increased in parallel with the cytochrome b_6f complex (Leong and Anderson, 1984; Chow and Anderson, 1987; Chow and Hope, 1987; Evans, 1987; Burkey and Wells, 1996).

Pronounced photosynthetic stoichiometry adjustments have also been observed in response to light qualities preferentially exciting either PSII–LHCII ('PSII light') or PSI ('PSI light'). Due to its large complement of chlorophyll *b*-containing LHCII proteins, which absorb light between wavelengths of ~450 nm and 640 nm more efficiently than the photosynthetic reaction centres only binding chlorophyll *a*, PSII tends to use these wavelengths more efficiently than PSI. On the other hand, PSII is much less efficient in using wavelengths >680 nm than PSI, which can use wavelengths even beyond 700 nm to drive photochemistry efficiently. Therefore, PSI may be preferentially excited in dense canopies, such as the lower vegetation levels in forests, where the light spectrum is depleted in photosynthetically active radiation between wavelengths of 400 nm and 700 nm, due to its strong absorbance by both photosystems and their antennae (reviewed by Dietzel *et al.*, 2008). To rebalance excitation rates of the two photosystems, 'PSI light' results in a repression of PSI contents, while PSII contents are increased. 'PSII light' induces the opposite response (reviewed by Schöttler and Tóth, 2014). Interestingly, while, large changes in photosystem stoichiometry, but no changes in cytochrome b_6f complex content occur in pea in response to the different light qualities (Chow *et al.*, 1990), in *A. thaliana* changes in cytochrome b_6f complex content are even more prominent than the photosystem stoichiometry adjustments. 'PSII light' results in an almost 50% increase in cytochrome b_6f complex levels (Dietzel *et al.*, 2011). To our knowledge, changes in ATP synthase content and activity have not been studied in response to different light qualities.

In summary, in response to changing metabolic ATP and NADPH demands during environmental fluctuations and leaf development, strong adjustments of the cytochrome b_6f complex and ATP synthase are observed. These changes in complex content and activity closely correlate with linear electron flux and leaf assimilation capacity, suggesting that the cytochrome b_6f complex and ATP synthase are the predominant points of photosynthetic flux control.

Co-regulation of ATP synthase and the cytochrome b_6f complex ensures the precise balancing of the photosynthetic proton circuit

A predominant role for the cytochrome b_6f complex in photosynthetic flux control was ultimately confirmed by the

specific inhibition of its activity with 2,5-dibromo-6-methyl-3-isopropyl-1,4-benzochinon (DBMIB; Kirchhoff *et al.*, 2000) and by the genetic repression of its contents via antisense and RNA interference (RNAi) approaches against its essential nuclear-encoded subunits PETC (the Rieske 2Fe2S protein; Price *et al.*, 1995; Yamori *et al.*, 2011; Hojka *et al.*, 2014) and PETM (Hojka *et al.*, 2014). In all cases, linear electron flux and leaf assimilation decreased linearly with cytochrome b_6f complex content and activity. The contribution of ATP synthase to photosynthetic flux control was elucidated with antisense approaches against its essential nuclear-encoded subunits (Rott *et al.*, 2011; Yamori *et al.*, 2011). Again, a very close correlation between ATP synthase activity, linear electron flux, and leaf assimilation was observed, pointing towards a major role for ATP synthase in photosynthetic flux control. Therefore, photosynthesis is co-limited by the cytochrome b_6f complex and ATP synthase.

The importance of the parallel adjustment of both complexes can be studied in transgenic plants suffering from a specific defect in the biogenesis or stability of one of the two complexes, so that their co-regulation is disturbed. This can severely compromise plant fitness. Light intensities, which are still limiting for a wild-type plant with perfect co-regulation of the cytochrome b_6f complex and ATP synthase and do not result in the induction of photoprotective mechanisms (Fig. 2A), can result in severe photosynthetic defects and strong phenotypes in such transformants.

In transformants with an impaired biogenesis or reduced stability of chloroplast ATP synthase (Rott *et al.*, 2011), an excess amount of the cytochrome b_6f complex, relative to ATP synthase, can result in a strong overacidification of the thylakoid lumen due to higher proton influx than efflux rates through ATP synthase already at light intensities which are still limiting for the wild type (Fig. 2B). The activation of qE already in limiting light severely decreases the quantum efficiency of photosynthetic CO_2 fixation and thereby strongly restricts plant growth under low or fluctuating light conditions. Because the production of both ATP and NADPH is slowed down due to photosynthetic control of plastoquinol re-oxidation, CO_2 assimilation by the Calvin–Benson cycle is decreased. Such a situation may also occur in some species such as sunflower in response to drought stress, resulting in a strong repression of ATP synthase (Tezara *et al.*, 1999).

The opposite situation, with an excess amount of ATP synthase relative to the cytochrome b_6f complex, is much more detrimental for the plant (Fig. 2C). Therefore, it has so far only been observed in transformants suffering from a decreased accumulation of the nuclear-encoded subunits of the cytochrome b_6f complex, but not as part of environmental or developmental acclimation responses in higher plants. The decreased accumulation of the cytochrome b_6f complex restricts linear electron transport and the formation of the pmf across the thylakoid membrane, because the proton efflux capacity through ATP synthase exceeds proton influx into the lumen by linear electron flux even under light-saturated conditions. These plants fail to reach the threshold level of lumen acidification required to activate fully photoprotective mechanisms such as qE and photosynthetic control at the cytochrome b_6f complex (Price *et al.*, 1995; Yamori *et al.*, 2011; Hojka *et al.*, 2014). Because of the restriction of linear electron flux at the cytochrome b_6f complex and the compromised induction of qE, the PSII acceptor side becomes over-reduced. This results in a massive generation of ROS by PSII, which in the most extreme case may trigger cell death pathways, resulting in large necrotic areas on leaves (Hojka *et al.*, 2014). Also, because the production of both ATP and NADPH is slowed down, CO_2 assimilation by the Calvin–Benson cycle is decreased.

In conclusion, a precise co-regulation of ATP synthase and the cytochrome b_6f complex is essential to ensure an optimized balance between light utilization for photosynthesis and growth and harmless dissipation of excess light by photoprotective mechanisms. Therefore, in this review, we focus on the mechanisms resulting in altered accumulation levels of these two complexes. We will begin with a detailed description of the structure and function of the cytochrome b_6f complex and ATP synthase, and will then focus on the signals and mechanisms, by which the stoichiometry adjustments are achieved.

Structure and function of the cytochrome b_6f complex and chloroplast ATP synthase

Cytochrome b_6f *complex*

The cytochrome b_6f complex is the smallest redox-active complex of the photosynthetic electron transport chain. Its structure has been elucidated in the cyanobacteria *Mastigocladus laminosus* (Kurisu *et al.*, 2003) and *Nostoc* sp. PCC 7120 (Baniulis *et al.*, 2009), and in the eukaryotic alga *Chlamydomonas reinhardtii* (Stroebel *et al.*, 2003). The cytochrome b_6f complex functions as a dimer with a total molecular mass of ~220 kDa, and each monomer consists of eight different subunits (Cramer *et al.*, 2006; Baniulis *et al.*, 2009). An overview of all subunits of the cytochrome b_6f complex and their function is provided in Table 1.

The small M subunit (PETM) and the Rieske 2Fe2S protein (PETC) are nuclear encoded, while the other six subunits are encoded in the chloroplast genome (plastome). In some cases, ferredoxin-$NADP^+$-oxidoreductase (FNR) binds to the stromal surface of a subpopulation of the cytochrome b_6f complex (Zhang *et al.*, 2001). This may alter the distribution of the cytochrome b_6f complex between stacked grana thylakoids and the unstacked stroma lamellae, because, after binding of the FNR, the cytochrome b_6f complex becomes too large to fit into the partition gap between the grana stacks. Complexes located in the unstacked membranes may not be available for rapid linear electron transport, due to inefficient long-distance diffusion of plastoquinone in the thylakoid membrane and of plastocyanin in the lumen (reviewed by Albertsson, 2001; Kirchhoff, 2014). Thereby, FNR binding to the cytochrome b_6f complex may rapidly down-regulate linear electron flux, and the cytochrome b_6f complexes redistributed into the stroma lamellae may function in cyclic electron flux (Szymanska *et al.*, 2011).

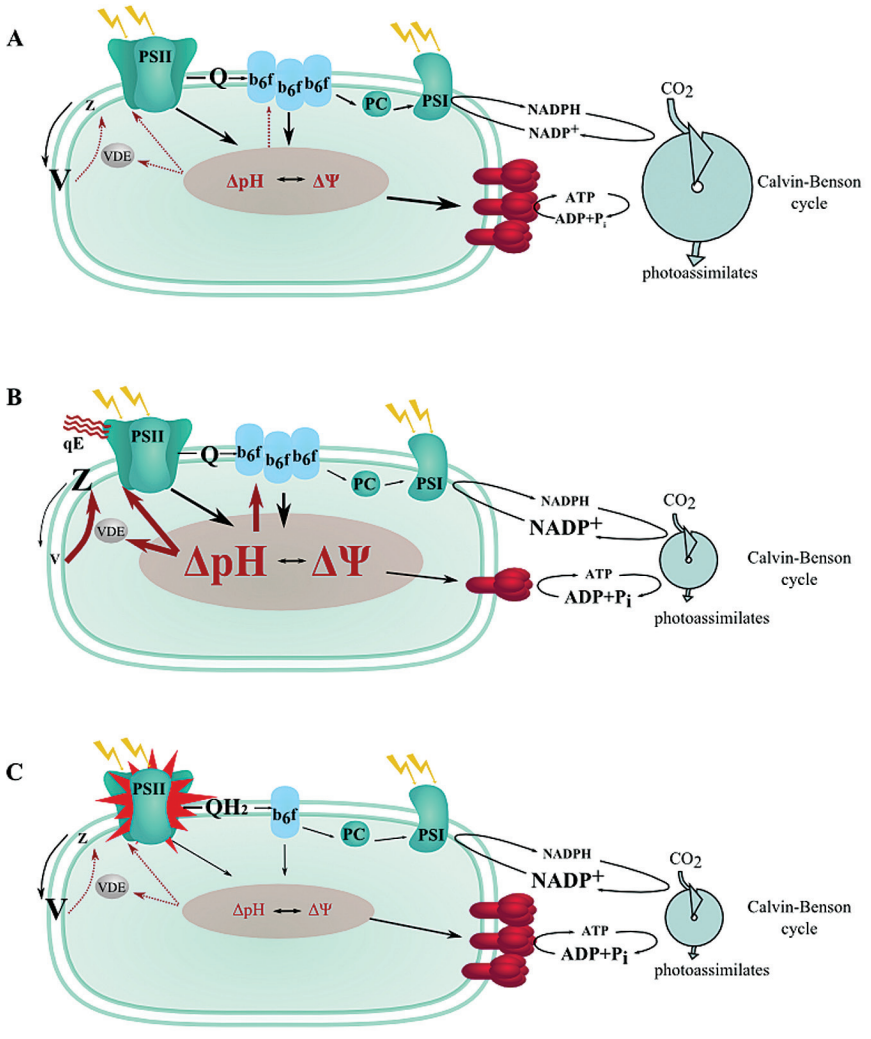

Fig. 2. Impaired co-regulation of the cytochrome b_6f complex and ATP synthase contents severely impairs photosynthesis and plant viability. In (A), the situation in wild-type plants is shown. Cytochrome b_6f complex and ATP synthase contents are strictly co-regulated to keep the pmf across the thylakoid membrane in a range which allows efficient ATP synthesis under light-limited conditions, but only weakly initiates photoprotective mechanisms such as qE (thylakoid lumen pH-dependent responses are indicated by the dotted red arrows). In (B), the situation for a transformant with an impaired biogenesis or reduced stability of chloroplast ATP synthase is shown. Here, an excess amount of cytochrome b_6f complex, relative to ATP synthase, allows a higher proton influx into the lumen by linear electron transport, than can be used by ATP synthase. This results in an overacidification of the thylakoid lumen already at light intensities which are still limiting for the wild type. As a consequence, a strong activation of photoprotective mechanisms such as qE and photosynthetic control of plastoquinol re-oxidation at the cytochrome b_6f complex (indicated by bold red arrows) occurs, which strongly reduces the quantum efficiency of photosynthesis. Because the production of both ATP and NADPH is slowed down, CO_2 assimilation by the Calvin–Benson cycle is decreased (indicated by the smaller size of the circle symbolizing the Calvin–Benson cycle). In (C), the situation for a transformant with an impaired biogenesis or reduced stability of the cytochrome b_6f complex is shown. An excess amount of ATP synthase, relative to the cytochrome b_6f complex, results in a higher proton efflux rate from the lumen than proton influx via linear electron transport, so that the threshold level of lumen acidification required for the activation of photoprotective mechanisms such as qE and photosynthetic control of plastoquinol re-oxidation at the cytochrome b_6f complex is not reached. As a consequence, PSII is destroyed by a massively increased production of ROS (indicated by the exploding structure), due to the over-reduction of the PSII acceptor side and the deficiency in qE induction. Because the production of both ATP and NADPH is slowed down, CO_2 assimilation by the Calvin–Benson cycle is decreased (indicated by the smaller size of the circle symbolizing the Calvin–Benson cycle). For details, please refer to the text.

Except for the FNR and the small chloroplast-encoded L subunit (PetL), which is located at the periphery of the complex and only plays a role in complex stability (Schöttler *et al*, 2007), all other subunits are essential for the function and accumulation of the cytochrome b_6f complex (Bruce and Malkin, 1991; Kuras and Wollman, 1994; Hager *et al.*, 1999; Monde *et al.*, 2000; Schwenkert *et al.*, 2007; Hojka *et al.*, 2014). Plastoquinol oxidation occurs at the luminal plastoquinol-binding side, the electropositive side (Q_p-side). The binding of plastoquinol to the Q_p-side may be among the rate-limiting steps at the cytochrome b_6f complex (reviewed by Hasan and Cramer, 2012). When plastoquinol has reached the Q_p-side, its first electron is transferred via the Rieske protein (PETC) and cytochrome *f* (PetA) to plastocyanin. The Rieske protein consists of an N-terminal membrane anchor domain and a larger luminal domain binding the 2Fe2S cluster. To transfer electrons from the Q_p-side to the covalently bound haem in cytochrome *f*, the luminal domain of the Rieske protein needs to move from its proximal position close to the Q_p-side to a position which is close enough to the haem

on cytochrome *f* to allow forward electron transfer (de Vitry *et al.*, 2004; Hasan *et al.*, 2013). The movement of the Rieske protein may be another rate-limiting step determining the slow enzymatic turnover rate of the cytochrome b_6f complex.

Plastoquinol oxidation by the Rieske protein is coupled to the release of both of its protons into the thylakoid lumen and generates a semiquinone radical, which acts as a strong reductant and injects its second electron into the Q-cycle (reviewed by Cramer and Zhang, 2006). Then, the fully oxidized plastoquinone dissociates from the Q_p-side. The second electron is transferred via the low potential and high potential haems, which are non-convalently bound to cytochrome b_6, to a second plastoquinone-binding site located on the stromal or electronegative side (n-side) of the cytochrome b_6f complex. Therefore, this second quinone-binding site is called the Q_n-side. Oxidation of a second plastoquinol at the Q_p-side provides the second electron for the full reduction of the semiquinone molecule bound to the Q_n-side, which upon reduction takes up two protons from the stroma. This plastoquinol then diffuses towards the Q_p-side, where it is re-oxidized, releasing both of its protons into the lumen and sending one electron forward towards plastocyanin and PSI, while the second electron is re-injected into the Q-cycle.

Subunit IV, the product of the *petD* gene, does not stably bind any redox-active cofactors and is believed to have mainly a scaffold function, but also forms part of the Q_p-side together with cytochrome b_6 (Cramer *et al.*, 2006). In cytochrome bc_1 complexes in respiratory electron transport chains, subunit IV and cytochrome b_6 form one large protein, and the split into two proteins is one of the distinguishing features of cytochrome b_6f complexes. Another unique feature of the cytochrome b_6f complex is the presence of the haem c_i, whose function is still enigmatic. Haem c_i is located close to the stromal surface of cytochrome b_6. It is covalently bound by an unusual single thioether bond to a cysteine on cytochrome b_6, and may allow a two-electron reduction of the plastoquinone in the Q_n-side, thereby preventing the accumulation of the reactive plastosemiquinone radical (Baymann *et al.*, 2007). Alternatively, haem c_i may function in cyclic electron flux, either as an electron acceptor from FNR bound to the stromal side of the b_6f complex or even by an independent pathway (Kurisu *et al.*, 2003; Stroebel *et al*, 2003).

Dimerization of the cytochrome b_6f complex mainly occurs through monomer–monomer interactions of the transmembrane helices of cytochrome b_6 and the transmembrane domain of the Rieske protein. On the opposite side to the monomer–monomer interface of the cytochrome b_6f complex, four small subunits with molecular masses of 3–4 kDa are bound to each monomer. They form single transmembrane helices and do not participate in the redox reactions of the complex. In contrast to the non-essential L subunit, in higher plants PetG, PETM, and PetN are all essential for cytochrome b_6f complex assembly and stability (Hager *et al.*, 1999; Schwenkert *et al.*, 2007; Hojka *et al.*, 2014).

Chloroplast ATP synthase

Chloroplast F_0-F_1-ATP synthase is exclusively localized in the unstacked parts of the thylakoid membrane, the stroma lamellae and end membranes, because its bulky catalytic head (see below) sterically excludes the enzyme complex from the grana stacks (Miller and Staehelin, 1976; Daum *et al.*, 2010). It utilizes the pmf across the thylakoid membrane to drive ATP synthesis. ATP synthase differs from all other photosynthetic complexes in that not all of its subunits are present in a 1:1 stoichiometry. No precise structure of the entire complex has been established to date, and not all catalytic properties of the enzyme are understood in detail. Like all F_0F_1-ATP synthases, chloroplast ATP synthase can be easily dissociated

Table 1. *List of structural subunits of the cytochrome b_6f complex*

Information is provided on the protein name, whether it is encoded in the nucleus (N) or chloroplast (C), the gene locus number in both *A. thaliana* and *C. reinhardtii*, alternative names, and gene function.

Name	Gene locus	Alternative name/orthologues	Function
PetA (C)	ATCG00540 ChreCp001	Cytochrome *f*	Essential subunit, involved in electron transfer
PetB (C)	ATCG00720 ChreCp008	Cytochrome b_6	Essential subunit, involved in electron transfer
PETC (N)	AT4G03280 Cre11.g467689	Rieske 2Fe2S subunit	Essential subunit, involved in electron transfer
PetD (C)	ATCG00730 ChreCp002	Subunit IV	Essential subunit
PetG (C)	ATCG00600 ChreCp045	–	Essential subunit
PetL (C)	ATCG00590 ChreCp068	–	Non-essential subunit, required for cytochrome b_6f complex stability
PETM (N)	AT2G26500 Cre12.g546150	–	Essential subunit
PetN (C)	ATCG00210 Cre16.g650100	–	Essential subunit

into two subcomplexes: the large membrane-extrinsic, soluble CF_1 moiety, the 'coupling factor', catalyses the formation of ATP and H_2O from ADP and P_i. The partly membrane-intrinsic, hydrophobic CF_0 moiety has a double function, in that it both forms a stator to fix the catalytic part of the CF_1 moiety and also converts the pmf across the thylakoid membrane into a molecular rotation, which is transmitted to CF_1 via a central stalk structure (reviewed by von Ballmoos *et al.*, 2009).

An overview of all subunits of the chloroplast ATP synthase and their function is provided in Table 2. CF_1 is composed of five different subunits: AtpA (the chloroplast-encoded α-subunit), AtpB (the chloroplast-encoded β-subunit), ATPC (the nuclear-encoded γ-subunit), ATPD (the nuclear-encoded δ-subunit), and AtpE (the chloroplast-encoded ε-subunit). They accumulate in the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. The $\alpha_3\beta_3$ subcomplex forms a hexamer with three catalytic nucleotide-binding sites, each located at the αβ interfaces, which cycle between different conformational states (see below). These conformational changes are driven by the rotation of the central stalk, a rotor shaft formed by the ε- and the γ-subunit, relative to the catalytic hexamer.

The central stalk is associated with a membrane-intrinsic ring structure, which is part of CF_0. In photosynthetic eukaryotes, this ring is composed of 14 c-subunits encoded by the chloroplast *atpH* gene (Seelert *et al.*, 2000). In addition to the 14 c-subunits, the CF_0 moiety consists of the three membrane-intrinsic subunits a (AtpI, plastid-encoded), b (AtpF, plastid-encoded), and b' (ATPG, nuclear-encoded). The highly hydrophobic a-subunit of CF_0 directly flanks the c_{14} ring and contains two half channels for proton movements. The first channel allows protons to enter into CF_0 from the luminal side and ends within the membrane directly in front of an acidic amino acid side chain of a c-subunit. Upon protonation of the acidic side chain, the c-subunit moves by ~26 ° into the lipid phase surrounding the c-ring, bringing the non-protonated acidic side chain of the next c-subunit in the ring in front of the luminal half channel, so that the protonation–movement cycle can be repeated (reviewed by von Ballmoos *et al.*, 2009). After an almost full 360 ° rotation of the c-ring, the protonated amino acid approaches the exit half channel on the a-subunit, allowing the proton to leave ATP synthase towards the stroma. In total, 14 protonation events are needed for the c-ring to complete a full 360 ° rotation, which is transmitted via the central stalk to the catalytic $\alpha_3\beta_3$ hexamer.

To counteract a rotation of the catalytic hexamer together with the central stalk, a peripheral stator formed by the membrane-extrinsic α-helical domains of the two CF_0 subunits b and b' as well as the CF_1 subunit δ locks $\alpha_3\beta_3$. Therefore, the catalytic hexamer cannot rotate itself, but instead undergoes conformational changes by the shifting position of the γ-subunit relative to the three catalytic centres. This catalytic cycle is called the rotational binding change mechanism of ATP synthesis (reviewed by Boyer, 1993; Cross, 2000; Junge *et al.*, 2009). Each catalytic centre proceeds through a cycle of conformational changes. In its simplest description, the cycle begins with the binding of P_i and ADP to the open state of the catalytic centre. The bulge of the γ-subunit is always facing towards this conformation of the catalytic centre (Junge

Table 2. *List of structural subunits of chloroplast ATP synthase*

Information is provided on the protein name, whether it is encoded in the nucleus (N) or chloroplast (C), the gene locus number in both *A. thaliana* and *C. reinhardtii*, alternative names, and gene function.

Name	Gene locus	Alternative name/orthologues	Function
AtpA (C)	ATCG00120 ChreCp050	CF_1 α-subunit	Essential catalytic subunit of CF_1
AtpB (C)	ATCG00480 ChreCp058	CF_1 β-subunit	Essential catalytic subunit of CF_1
ATPC1 (N)	AT4G04640 Cre06.g259900	CF_1 γ-subunit	Essential central stalk subunit, redox-regulated
ATPC2 (N)	Only in higher plants: AT1G15700	CF_1 γ-subunit	Non-essential central stalk subunit, not redox-regulated, may mainly function in non-photosynthetic tissues
ATPD (N)	AT4G09650 Cre11.g467569	CF_1 δ-subunit	Essential subunit, attachment of CF_1 to the stator
AtpE (C)	ATCG00470 ChreCp023	CF_1 ε-subunit	Essential central stalk subunit
AtpF (C)	ATCG00130 ChreCp054	CF_0 subunit b CF_0 subunit I	Essential subunit of CF_0, forms the stator together with ATPG
ATPG (N)	AT4G32260 Cre11.g481450	CF_0 subunit II CF_0 subunit b'	Essential subunit of CF_0, forms the stator together with AtpF
AtpH (C)	ATCG00140 ChreCp053	CF_0 subunit c CF_0 subunit III	Essential subunit of CF_0, part of the molecular rotor, generates torque
AtpI (C)	ATCG00150 ChreCp062	CF_0 subunit a CF0 subunit IV	Essential subunit of CF_0, proton translocation through two halfchannels

et al., 2009). Then, after an ~120 ° rotation of the central stalk, the catalytic centre enters a conformation in which ADP and P_i are loosely bound. A further 120 ° rotation of the central stalk ultimately produces a conformational state with tight binding of both substrates, which allows the formation of ATP. After ATP formation, the central stalk can rotate by another 120 °, completing the catalytic cycle by the return of the catalytic centre to the open conformation, which releases ATP and binds ADP and P_i again. ATP formation in the tightly bound conformation of the catalytic centre becomes possible because the previous reaction steps result in the exclusion of water molecules from the catalytic side, so that the formation of ATP and H_2O from ADP and P_i becomes favourable. The major energy-requiring step of this reaction cycle is not the formation of ATP itself, but rather the co-operative binding of the substrates to the catalytic nucleotide-binding site, and the product release once the catalytic cycle is completed (reviewed by Cross, 2000).

Chloroplast ATP synthase activity in higher plants is strictly regulated. In darkness, ATP synthase is inactivated, to prevent ATP hydrolysis when the pmf across the thylakoid membrane is low. This inactivation is mainly due to redox regulation of the conformation of the γ-subunit of CF_1. In darkness, when two cysteines in the γ-subunit are oxidized and form a disulphide bridge, the activity of ATP synthase is strongly inhibited (reviewed by Hisabori *et al.*, 2013). Also in the light, up to 50% of ATP synthase can be inactive, so that an up to 50% repression in ATP synthase content by transgenic approaches can be fully compensated by the activation of the previously inactive enzyme population (Rott *et al.*, 2011). This inactive population is not attributable to an incomplete thioredoxin-mediated reduction of the redox-regulated cysteines; it is probably due to the reversible phosphorylation of the catalytic β-subunit of CF_1 (Rott *et al.*, 2011). While the phosphorylation states of two of these sites change greatly between day and night and thus could be involved in the nocturnal inactivation of ATP synthase (del Riego *et al.*, 2006; Reiland *et al.*, 2009), a third phosphorylation site may be involved in the binding of 14-3-3 proteins, which can drastically reduce ATP synthase activity, at least *in vitro* (Bunney *et al.*, 2001).

In plastids of heterotrophic tissues such as roots and etioplasts, functional ATP synthases are also found. However, in contrast to the chloroplast ATP synthases in leaves, they contain an alternative isoform of the γ-subunit, ATPC2. ATPC2 is not redox regulated, because the redox potential of its two cysteines is substantially higher than in ATPC1, so that they stay reduced in darkness. These ATP synthases may hydrolyse ATP in darkness, to generate a pmf to sustain protein translocation and other processes (Kohzuma *et al.*, 2012).

Photosynthetic complex biogenesis: an overview

Except for the nuclear-encoded LHC proteins, all photosynthetic complexes are chimeras composed of subunits encoded in the nucleus and in the chloroplast. Therefore, photosynthetic stoichiometry adjustments require the co-ordination of gene expression and protein biosynthesis between these two compartments (summarized in Fig. 3). To initiate these adjustments, environmental parameters and the metabolic demand of the plant need to be perceived in the photosynthetically active mesophyll cells. Both the metabolic demand of the mesophyll cell itself and the demand of sink tissues for photoassimilates have to be integrated (see 'Signals regulating photosynthetic complex accumulation'). Then, these signals need to initiate changes in photosynthetic complex biogenesis and turnover, which can potentially take place at any of the numerous steps involved in these processes.

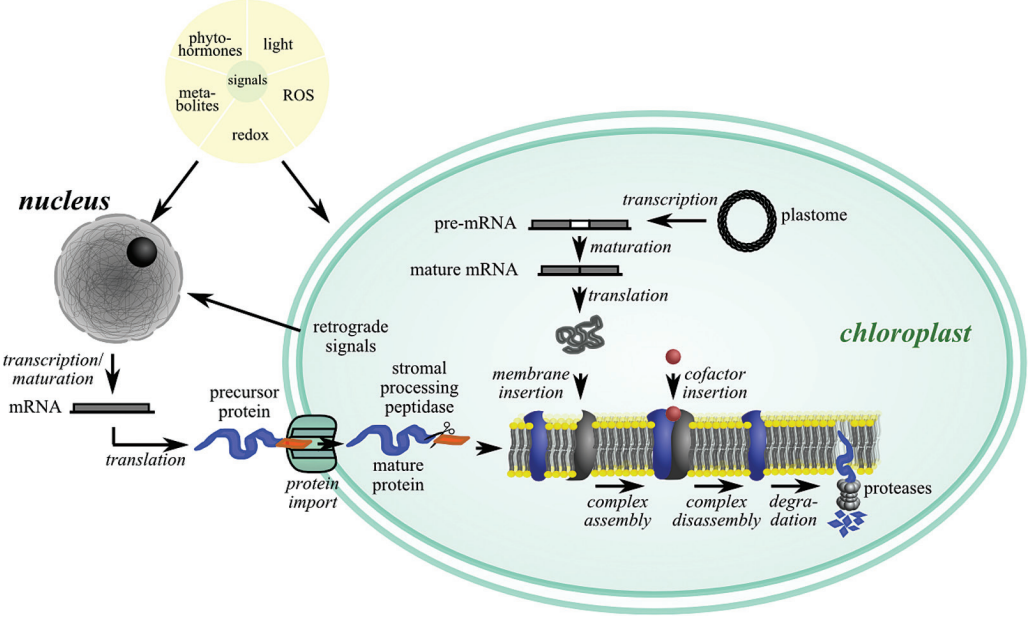

Fig. 3. Overview of the different levels of regulation in photosynthetic complex biogenesis: For details, please refer to the sections 'Photosynthetic complex biogenesis: an overview' and 'Signals regulating photosynthetic complex accumulation'.

In the case of nuclear-encoded genes, translation occurs in the cytosol, followed by the targeting of the precursor proteins into the chloroplast via a specific N-terminal signal peptide, the chloroplast transit peptide, which labels the protein for import across the outer and inner envelope membranes into the chloroplast (reviewed by Jarvis, 2008; Shi and Theg, 2013). In the chloroplast, the transit peptide is usually cleaved off by the stromal processing peptidase, and the mature protein remains in the stroma, for example in the case of Rubisco or the nuclear-encoded CF_1 subunits of ATP synthase (Fig. 3). Nuclear-encoded proteins, which are further targeted towards the thylakoid membrane or the lumen, bear a bipartite signal peptide, whose second part acting as a thylakoid signal peptide usually becomes functional after the cleavage of the envelope transit peptide by the stromal processing peptidase. Thylakoid membrane insertion either is mediated by a 'spontaneous' mechanism, which supports the membrane insertion of several small subunits of the photosynthetic complexes and of subunit b of ATP synthase (see 'Biogenesis of chloroplast ATP synthase'), or is supported by the chloroplast signal recognition particle pathway (reviewed by Aldridge *et al.*, 2009, Richter *et al.*, 2010). Translocation of the nuclear-encoded proteins into the thylakoid lumen occurs either via the chloroplast Sec pathway, which transports unfolded proteins and is dependent on nucleotide hydrolysis, or by the chloroplast Tat pathway, which transports folded proteins through the thylakoid membrane and is pmf dependent (reviewed by Albiniak *et al.*, 2012). The thylakoid transit peptide is usually removed by the thylakoid processing peptidase, which is located on the luminal surface of the thylakoid membrane.

In the chloroplasts of higher plants, genes can be transcribed by three different RNA polymerases. Two RNA polymerases, called RPOTp and RPOTmp, are nuclear encoded, monomeric, and evolutionarily related to bacteriophage RNA polymerases. The third RNA polymerase, called the 'plastid-encoded RNA polymerase' (PEP), consists of several chloroplast-encoded subunits and is related to eubacterial RNA polymerases (reviewed by Liere *et al.*, 2011). In higher plants, its activity is regulated by at least six nuclear-encoded sigma factors (reviewed by Schweer *et al.*, 2010; Lerbs-Mache, 2011; Lyska *et al.*, 2013). Additionally, >10 other nuclear-encoded regulatory proteins associate with the PEP and, together, they constitute the 'transcriptionally active chromosome complex'. They may have structural and regulatory functions, thus enabling nuclear control of chloroplast gene expression (Steiner *et al.*, 2011).

Most plastid-encoded genes are co-transcribed and form polycistronic mRNAs. They may also contain introns (Fig. 3). Many polycistronic transcripts undergo a complex post-transcriptional maturation, which may depend on the developmental and physiological state of the plant: the most important processes are the splicing of introns, the cleavage of polycistronic units into smaller, mono- or dicistronic transcripts, and trimming at both the 5′ and the 3′ end of the mRNAs. In a few cases, individual nucleotides are also modified via RNA editing (reviewed by Bollenbach *et al.*, 2007; Schmitz-Linneweber and Barkan, 2007; Lyska *et al.*, 2013). Even after the mature mRNA has been formed, its stability may depend on its binding to specific nuclear-encoded proteins, which protect the mRNA against degradation.

Once a mature mRNA has been formed, its translation by the 70S ribosomes of the chloroplast often requires additional transcript-specific nuclear-encoded translation initiation factors. At least in *C. reinhardtii*, gene expression is almost exclusively controlled at the level of transcript stability and translation initiation, while transcriptional regulation and mRNA maturation seem to be less important (Choquet *et al.*, 2001; Eberhard *et al.*, 2002; Raynaud *et al.*, 2007; Boulouis *et al.*, 2011). However, in contrast to the situation in higher plants, green algae such as *C. reinhardtii* contain only the eubacterial-type RNA polymerase with only a single sigma factor, indicating that substantial differences in the complexity of transcriptional regulation may exist between algae and higher plants (Carter *et al.*, 2004; Bohne *et al.*, 2006).

Thylakoid membrane insertion of plastid-encoded proteins can occur post-translationally, for example in the case of the cytochrome b_6f complex subunits PetB and PetD (see 'Biogenesis of the cytochrome b_6f complex'), or in the case of the b-subunit of CF_0 (see 'Biogenesis of chloroplast ATP synthase'). In some cases, post-translational membrane insertion is supported by the Sec translocase. Other thylakoid-intrinsic proteins are co-translationally inserted into the membrane. During this co-translational membrane insertion, ribosomal pausing may allow cofactor binding to the nascent polypeptide chain. A strict co-ordination of complex assembly and cofactor biosynthesis is required, because the free forms of some cofactors can function as photosensitizers generating ROS.

Complex assembly and cofactor attachment are often supported by specific auxiliary proteins. Their functions have been studied in most detail for the biogenesis of the two photosystems. There, auxiliary proteins support the co-translational membrane insertion of the chloroplast-encoded subunits and attach the redox-active cofactors and specific pigments to the nascent polypeptide chains. Other auxiliary proteins process newly inserted subunits, stabilize assembly intermediates, and attach peripheral subunits to the nascent complex (reviewed by Mulo *et al.*, 2008; Schöttler *et al.*, 2011; Nickelsen and Rengstl, 2013). It has been speculated that the availability of auxiliary proteins could contribute to the control of PSI biogenesis (reviewed by Schöttler *et al.*, 2011). Finally, photosynthetic complex turnover and degradation could be highly regulated processes (see 'Photosynthetic complex lifetimes and turnover' and 'Degradation of chloroplast ATP synthase and the cytochrome b_6f complex'). Ultimately, the interplay of all these levels of regulation enables plants to rebalance photosynthetic complex contents and ATP and NADPH production to altered environmental conditions or metabolic states of the plant.

Signals regulating photosynthetic complex accumulation

Hundreds of publications have analysed changes in (photosynthetic) gene expression during plant development,

in response to different environmental conditions and to the application of external effectors. In these publications, mainly changes in mRNA accumulation, and less frequently also in transcription rates have been determined. However, it is well established that even relatively large changes in mRNA accumulation both in the chloroplast and in the nucleus do not necessarily result in comparable changes in photosynthetic complex contents (see 'Regulation of cytochrome b_6f complex accumulation' and 'Regulation of ATP synthase accumulation' on candidates for limiting factors in the biogenesis of the cytochrome b_6f complex and ATP synthase). Therefore, instead of summarizing current knowledge on signals resulting in changes in gene expression, we here focus on the developmental conditions and environmental perturbations summarized in 'Long-term responses of the photosynthetic apparatus during leaf ontogenesis and environmental acclimation', which have already been demonstrated to initiate large adjustments in the contents of both complexes. Then, we go backwards towards possible signals initiating the adjustments.

The studies summarized in 'Long-term responses of the photosynthetic apparatus during leaf ontogenesis and environmental acclimation' indicate that signals balancing the source capacity of leaves to the photoassimilate demand of sink tissues have a strong effect on the biogenesis of both complexes. Because a low sink strength will reduce photoassimilate transport efficiency from source to sink tissues, the accumulation of photoassimilates such as sucrose and hexoses in source leaves could generate a metabolic signal in the mesophyll tissue, which represses photosynthetic activity (see below). Light quality and light intensity also strongly affect the accumulation of both the cytochrome b_6f complex and ATP synthase. They could either be directly perceived via photoreceptors, or they could result in an altered redox state of the photosynthetic apparatus, which then might trigger adjustments of the cytochrome b_6f complex and ATP synthase contents (see 'Light and redox signals' below). In *C. reinhardtii*, the production of nitric oxide (NO) has been shown to activate pathways resulting in the degradation of the cytochrome b_6f complex in response to nutrient limitations (see later). In all cases, because stoichiometry adjustments probably require changes in photosynthetic gene expression both in the chloroplast and in the nucleus, either the relevant signals have to be perceived in both compartments to adjust the gene expression, or a precise communication between the chloroplast and nucleus is required via retrograde and anterograde signals. Also, evidence exists for a complex cross-talk between the signals associated with the metabolic state of the cell and the redox state of the chloroplast with signals arising from ROS and phytohormones (Gläßer *et al.*, 2014).

Metabolic signals

The role of the metabolic state of the source leaf in photosynthetic gene expression has been studied for >20 years. Defects in photoassimilate export from source leaves due to the genetic manipulation of phloem loading via the expression of an apoplastic invertase were shown to result in a rapid repression of photosynthesis (e.g. von Schaewen *et al.*, 1990; Stitt *et al.*, 1991; Schöttler *et al.*, 2004). Also sugar feeding to detached leaves and autotrophic cell cultures was shown to repress photosynthesis massively (e.g. Krapp *et al.*, 1993; Kilb *et al.*, 1996). When *A. thaliana* seedlings are grown on synthetic media containing high concentrations of glucose or sucrose, the development of the photosynthetic apparatus in the cotyledons can be completely repressed (e.g. Zhou *et al.*, 1997). Under physiological conditions, a high accumulation of photoassimilates in source leaves will occur whenever the source capacity exceeds the sink demand.

The metabolic down-regulation of photosynthesis is related to the accumulation of photoassimilates such as sucrose, glucose, and fructose, both in the apoplast of source leaves and in the cytosol of mesophyll cells. Their accumulation is perceived by different 'sugar-sensing' mechanisms and then switches photosynthetic gene expression from a 'famine mode' with a requirement for high assimilation capacity to a 'feast mode', where the plant demand for photoassimilates is largely saturated (reviewed by Koch, 1996; Smeekens, 2000; Pego *et al.*, 2000). Major nuclear-encoded target genes of 'sugar sensing' include the small subunit of Rubisco (RBCS) and other Calvin–Benson cycle enzymes, but also the Rieske protein (PETC), plastocyanin (PETE), and both the γ- and δ-subunit of chloroplast ATP synthase.

It is still a matter of debate how the leaf sugar status is perceived by the plant, and to what extent sugar signalling pathways interact with phytohormone response pathways. Because the biogenesis of the photosynthetic apparatus can be completely repressed in *A. thaliana* seedlings by growing them on high concentrations of either sucrose or glucose, mutant screens have been used to identify mutants which are either less sensitive or hypersensitive to the sugar repression of photosynthesis (e.g. Zhou *et al.*, 1997). With this approach, the cytosolic hexokinase isoform HXK1, which also associates with the outer envelope of the chloroplasts, has been identified as a major intracellular hexose sensor, but several alternative pathways to perceive sugars both within the cytosol and in the apoplast have also been proposed (reviewed by Hanson and Smeekens, 2009; Häusler *et al.*, 2014).

Besides playing a role in feedback adjustments of photosynthesis and leaf assimilation in response to a changing sink demand for photoassimilates, sugar sensing may also play an important role in the light acclimation of the photosynthetic apparatus. In high-light conditions, an increased assimilation rate will result in at least a transient accumulation of photoassimilates such as sucrose, glucose, and fructose in source leaves, which then may act as negative regulators especially of LHCII expression (Schmitz *et al.*, 2014): the *A. thaliana adg1-1* mutant deficient in ADP-glucose pyrophosphorylase cannot accumulate starch and therefore accumulates exceptionally high concentrations of sucrose and hexoses after a shift to high light. This results in a more pronounced repression of *LHCB1* gene expression than in the wild type (Schmitz *et al*, 2014).

The *adg1-1*/*tpt-2* double mutant is affected both in the ADP-glucose pyrophosphorylase and in the triose-phosphate/phosphate translocator in the inner chloroplast envelope

membrane, which is required for the export of triose-phosphates from the chloroplast during the day. It can accumulate neither starch nor soluble sugars (Schmitz *et al.*, 2012). Under high-light conditions, the LHCII contents in the double mutant remain unchanged, and large amounts of uncoupled LHCII accumulate in the thylakoid membrane, giving rise to a high chlorophyll fluorescence phenotype (Schmitz *et al.*, 2012). The *adg1-1*/*tpt-2* mutant also fails to increase the contents of the cytochrome b_6f complex and ATP synthase with the increasing growth light intensity. Instead, accumulation of the reaction centres of both photosystems is reduced, possibly due to oxidative stress. Surprisingly, the double mutant accumulates large amounts of free cytochrome b_{559} in its thylakoids (Schmitz *et al.*, 2012). After sugar feeding to the *adg1-1*/*tpt-2* double mutant, its high light acclimation is rescued.

This impaired high light acclimation is attributable to a massive deregulation of photosynthetic gene expression both in the nucleus and in the chloroplast. In contrast to the wild type and the *adg1-1* single mutant, neither sucrose nor glucose or fructose concentrations increase in the double mutant after the high light shift. This may explain why *LHCB1* expression is not repressed (Schmitz *et al.*, 2014). Also the expression of several plastome-encoded subunits of the photosystems and of the cytochrome b_6f complex gene *petD* and the two ATP synthase CF_0 genes *atpF* and *atpH* is strongly deregulated in the *adg1-1*/*tpt-2* double mutant (Häusler *et al.*, 2014; Schmitz *et al.*, 2014).

These data strongly indicate that sugars also modulate gene expression in the chloroplast. However, it is still unclear whether the sugar signals are perceived in the chloroplast itself, or transmitted from the cytosol to the plastome. Based on theoretical considerations and experimental observations, for a long time a direct sensing of non-phosphorylated sugars in the chloroplast has been considered to be unlikely. During the day, triose-phosphates are either quantitatively exported to the cytosol for sucrose biosynthesis or converted into starch or amino acids within the chloroplast, but not into free non-phosphorylated sugars. Therefore, only very low concentrations of sucrose and no non-phosphorylated hexoses are detected in the chloroplast stroma of barley (Winter *et al.*, 1993), spinach (Winter *et al.*, 1994), and tobacco (Heineke *et al.*, 1994). Depending on the balance between starch degradation and the export of the degradation products from the chloroplast to the cytosol, glucose and maltose may accumulate only during the night (reviewed by Fettke *et al.*, 2009). However, because their levels are not directly related to photosynthesis, they are unlikely to modulate photosynthetic gene expression in the chloroplast.

However, recently, clear evidence for sucrose uptake into chloroplasts, and its conversion into hexose-phosphates was presented. Both in tobacco and potato (*Solanum tuberosum*), the heterologous expression of a chloroplast-targeted fructosyltransferase from *Bacillus subtilis*, which converts sucrose into fructan, resulted in high level fructan accumulation in the chloroplast. This unequivocally confirms the presence of sucrose in the chloroplast (Gerrits *et al.*, 2001). One possibility is that sucrose and hexoses accumulating in the cytosol may diffuse through the envelope membranes into the chloroplast. In the case of high cytosolic glucose contents, glucose uptake into the chloroplast may occur via the glucose transporter normally involved in nocturnal glucose export from the chloroplast (Weber *et al.*, 2000). In the case of sucrose, however, no evidence for a specific transporter exists to date (Heldt and Sauer, 1971).

Recently, enzymatically active chloroplast isoforms of enzymes such as hexokinase (Giese *et al.*, 2005; Zhang *et al.*, 2010), fructokinase (Arsova *et al.*, 2010), and of an alkaline/neutral invertase (AtINVE; Vargas *et al.*, 2008) were identified. AtINVE constitutes ~20% of total leaf invertase activity and may function in the removal of sucrose diffusing into the plastid via hydrolytic cleavage, thereby maintaining a concentration gradient between the cytosol and the chloroplast (Vargas *et al.*, 2008). Subsequently, the chloroplast hexokinase and fructokinase may phosphorylate glucose and fructose. The hexose-phosphates may then be used as substrates for starch biosynthesis or other anabolic processes within the chloroplast. As part of these reactions or independently of them, sugar sensing in the plastid may occur, which then could directly modify chloroplast gene expression.

Intriguingly, the plastidic hexokinase isoform has been implicated in the sugar sensing-mediated regulation of nuclear gene expression, suggesting a complex role for retrograde signals from the chloroplast in the metabolic regulation of photosynthetic gene expression in the nucleus (Zhang *et al.*, 2010). Also the alkaline/neutral invertase may play a role in the regulation of photosynthetic gene expression (Tamoi *et al.*, 2010). The *A. thaliana sicy-192* mutant has a single point mutation in AtINVE, which does not alter the enzymatic properties of the enzyme, but results in slightly elevated accumulation and activity of the chloroplast invertase. *Sicy-192* seedlings are hypersensitive to external sucrose and do not develop a photosynthetic apparatus, and the expression of several nuclear-encoded photosynthesis-related genes is strongly repressed. Because this repression is unaltered in double mutants with additional defects in known cytosolic sugar-sensing components such as HKX1, the chloroplast invertase may constitute the first component of a novel sugar-sensing and signal transduction pathway. However, it cannot be fully excluded that the small increase in AtINVE activity in the *sicy-192* mutant may disturb chloroplast bioenergetics so that photosynthetic complex biogenesis is impaired (Tamoi *et al.*, 2010).

Recently, two fructokinase-like proteins, FLN1 and FLN2, have also been identified as components of the transcriptionally active chromosome in chloroplasts (Arsova *et al.*, 2010; Steiner *et al.*, 2011). Both proteins are catalytically inactive, but interact closely with a thioredoxin *z* (Arsova *et al.*, 2010). In transformants suffering from reduced accumulation of FLN1 or thioredoxin *z*, PEP-dependent chloroplast gene expression is massively disturbed, and thylakoid membrane accumulation is impaired (Arsova *et al.*, 2010). Therefore, the FLN proteins and thioredoxin *z* were originally suggested to couple chloroplast gene expression to the chloroplast redox state and photosynthetic electron transport. However, the complementation of knock-out mutants with

modified redox-inactive versions of FLP and thioredoxin *z*, respectively, almost completely rescued the plants, strongly indicating that redox regulation of the PEP is not the function of the FLN proteins and thioredoxin *z* (Wimmelsbacher and Börnke, 2014). Instead, even though they are catalytically inactive, FLN1 and FLN2 may function in fructose sensing in the chloroplast. Alternatively, they may just be structural components required for the normal assembly of PEP (Wimmelsbacher and Börnke, 2014).

Finally, changes in the cytosolic sugar state could be transmitted to the chloroplasts via a trehalose-6-phosphate-dependent mechanism. The cellular concentration of trehalose-6-phosphate closely follows that of sucrose in the cell (Lunn *et al.*, 2006), and has been shown to modulate starch breakdown in the chloroplast (Martins *et al.*, 2013).

Light and redox signals

The accumulation of the cytochrome b_6f complex responds strongly to different light intensities and light qualities (see earlier). However, it is difficult to dissect precisely the signal triggering these adjustments. In addition to metabolic signals, changes in light intensity and quality may be directly perceived by photoreceptors such as the different phytochromes and cryptochrome, which then could trigger changes in photosynthetic gene expression and complex synthesis. Alternatively, because all changes in light intensity and light quality will also alter the redox poise of the electron transport chain itself and of the chloroplast stroma, redox signals may trigger stoichiometry adjustments. For example, both a preferential excitation of PSI by far-red light and low light intensities equally exciting the two photosystems result in an oxidized pool and low accumulation levels of the cytochrome b_6f complex. On the other hand, both a preferential excitation of PSII in low light and high light conditions result in a reduction of the plastoquinone pool and in an increase in cytochrome b_6f complex content. The redox state of the plastoquinone pool is perceived by the occupancy of the Q_p-side of the cytochrome b_6f complex (Vener *et al.*, 1995; Zito *et al.*, 1999) and can result in the rapid activation of the thylakoid protein kinase STT7 in *C. reinhardtii* (Depege *et al.*, 2003) and STN7 in higher plants (Bonardi *et al.*, 2005). In the short term, the STT7/STN7 kinase reversibly phosphorylates LHCII, which then dissociate from PSII and are redistributed to PSI ('state transitions'; reviewed by Rochaix *et al.*, 2012).

However, STN7 also initiates photosynthetic complex stoichiometry adjustments in response to a long-term excitational imbalance between the photosystems (Bonardi *et al.*, 2005; Pesaresi *et al.*, 2009). Therefore, an STN7 mutant can be used as a control to distinguish between true light signals perceived via photoreceptors and redox signals perceived in the chloroplast. The use of photoreceptor mutants is much more problematic: a large number of different phytochrome and blue-light receptor isoforms exists, and mutants affected in some of these suffer from severe developmental defects, which may indirectly interfere with photosynthetic gene expression and complex accumulation (Fey *et al.*, 2005). Because the changes in cytochrome b_6f complex contents in response to changes in light quality are largely abolished in the STN7 mutant (Dietzel *et al.*, 2011), a major role for light signals perceived via photoreceptors in the adjustments appears to be unlikely. However, the level of the *petB* mRNA stability factor PRFB3 (see below, section 'Regulation of cytochrome b_6f complex accumulation') seems to be directly controlled by photoreceptors, and it has been suggested that drastic changes in PRFB3 abundance may alter cytochrome b_6f complex accumulation (Stoppel *et al.*, 2011).

Similar to the metabolic signals, the precise signal transduction cascades which initiate cytochrome b_6f complex stoichiometry adjustments in response to redox signals remain to be elucidated. Redox signals from the plastoquinone pool can alter photosynthetic gene expression within the chloroplast in an STN7-dependent manner. For example, an oxidized plastoquinone pool stimulates the expression of PSII reaction centre subunits in the chloroplast. When the plastoquinone pool is reduced, the expression of PSII reaction centre subunits is repressed, but the expression of the chloroplast-encoded PSI subunits is increased (Pfannschmidt *et al.*, 1999). The redox state of the plastoquinone pool is also transduced to the nucleus via a retrograde signal cascade, and modulates the expression of the different nuclear-encoded subunits of the photosystems there (Pfannschmidt *et al.*, 2001; Fey *et al.*, 2005). However, the redox regulation of the expression of the plastid- and nuclear-encoded subunits of the cytochrome b_6f complex has not been elucidated so far, and the subunits of the cytochrome b_6f complex have not been among the genes that most strongly respond to chloroplast redox signals (Fey *et al.*, 2005).

Biogenesis of the cytochrome b_6f complex

It is still largely unknown how the cytochrome b_6f complex is assembled. In bacteria and yeast mitochondria, the precise assembly sequence of the cytochrome bc_1 complexes of respiratory electron transport chains has been determined (reviewed by Zara *et al.*, 2009; Smith *et al.*, 2012). Even though the structures of the bacterial and mitochondrial cytochrome bc_1 complexes differ dramatically from each other and from the cytochrome b_6f complex, some basic assembly principles may be derived from these studies. In bacteria, whose bc_1 complexes are formed by only three subunits (Kleinschroth *et al.*, 2011), cytochrome c_1 binds to cytochrome *b*, which is followed by the insertion of the Rieske protein. Dimerization is the last step of the assembly process (Smith *et al.*, 2012). In mitochondria, whose bc_1 complexes consist of at least 10 different subunits, this general assembly sequence is conserved, even though some of the additional subunits may first bind to the Rieske protein and then assemble as a module together with the Rieske protein into the nascent complex (Zara *et al.*, 2009). Although a detailed investigation is still missing, the assembly of the cytochrome b_6f complex core could follow a similar pattern, with cytochrome b_6 and subunit IV assembling first, thus creating a structure similar to cytochrome *b* in bacteria. This could be followed by the insertion of cytochrome *f* and the Rieske protein. The binding sequence of the four small

subunits and of the additional cofactors remains enigmatic. A list of all auxiliary proteins involved in the biogenesis of the cytochrome b_6f complex is provided in Table 3.

Subunit synthesis and cofactor attachment

Best understood so far in cytochrome b_6f complex biogenesis are the mechanisms by which the individual precursor proteins and apoproteins of the three large redox-active subunits PetA, PetB, and PETC are inserted into the thylakoid membrane. Also the protein maturation and attachment of cofactors have been characterized in some detail. In all vascular plants, cytochrome b_6 (PetB) and subunit IV (PetD) are encoded by the last two genes of a large polycistronic unit also comprising the PSII subunit genes *psbB* (CP47), *psbT*, and *psbH* (Stoppel and Meurer, 2013). This polycistronic unit has a single transcription start site in front of the *psbB* gene and a single terminator behind *petD*. The large polycistronic transcript undergoes complex processing, including the excision of two group II introns from the coding regions of *petB* and *petD* and endonucleolytic cleavage. This produces monocistronic transcripts for each of the three PSII mRNAs and *petB*, a dicistronic *psbH–petB* transcript, and a larger dicistronic *petB–petD* transcript comprising both cytochrome b_6f complex subunit genes. In *A. thaliana*, free *petD* transcript does not accumulate, possibly because it is rapidly degraded after cleavage of the *petB–petD* intergenic region (Westhoff and Herrmann, 1988; Felder *et al.*, 2001; Meierhoff *et al.*, 2003). The pentatricopeptide repeat (PPR) protein HCF152 binds to the 5' end of the *petB*-containing transcripts and protects them from degradation by exonucleases. In the absence of HCF152, no cytochrome b_6f complex can accumulate (Meierhoff *et al.*, 2003; Pfalz *et al.*, 2009). In vascular plants, the 3' end of the monocistronic *petB* transcript is stabilized by the PRFB3 protein, in whose absence the cytochrome b_6f complex is specifically lost from thylakoid membranes. PRFB3 may play an additional role in translation initiation of the *petB* mRNA (Stoppel *et al.*, 2011).

Table 3. *List of auxiliary proteins involved in the biogenesis of the cytochrome* b_6f *complex*

Information is provided on the protein name, whether it is encoded in the nucleus (N) or chloroplast (C), the gene locus number in both *A. thaliana* and *C. reinhardtii*, alternative names, and gene function.

Name	Gene locus	Alternative name/orthologues	Function
CCB1 (N)	AT3G26710 Cre16.g662150		Haem c_i insertion into apocytochrome b_6
CCB2 (N)	AT5G52110 Cre12.g537850	HCF208	Haem c_i insertion into apocytochrome b_6
CCB3 (N)	AT5G36120		Haem c_i insertion into apocytochrome b_6
CCB4 (N)	AT1G59840 Cre08.g382300		Haem c_i insertion into apocytochrome b_6
CCDA (N)	AT5G54290 Cre07.g357800		Haem insertion into apocytochrome *f*: thiol-disulphide transporter
CCS1 (N)	AT1G49380 Cre13.g575000		Haem insertion into apocytochrome *f*: haem transfer across thylakoid membrane
CCS2 (N)	Locus not identified		Haem insertion into apocytochrome *f*, function unknown
CCS3 (N)	Locus not identified		Haem insertion into apocytochrome *f*, function unknown
CcsA (C)	ATCG01040 ChreCp028	Ycf5	Haem insertion into apocytochrome f: haem transfer and donor for haem ligation into apocytochrome *f*
Q	AT3G17930 Cre01.g000850	CPLD38	Assembly of PetD
FKBP13 (N)	AT5G45680		Regulation of the import of the Rieske protein, function questionable
HCF152 (N)	AT3G09650		Stabilizes *petB* 5'-UTR
HCF153 (N)	AT4G31560	CCS4 (*C. reinhardtii*)	Haem insertion into apocytochrome *f*: thiol-disulphide transporter across thylakoid membrane
HCF164 (N)	AT4G37200 Cre17.g702150	CCS5 (*C. reinhardtii*)	Haem insertion into apocytochrome *f*: thiol-disulphide transporter across thylakoid membrane
MCA1 (N)	Only in *C. reinhardtii*: Cre08.g358250		*petA* transcript accumulation and translation initiation
PRFB3 (N)	Only in higher plants: AT3G57190		Stabilizes 3' end of *petB*; translation initiation of *petB* mRNA
TCA1 (N)	Only in *C. reinhardtii*: Cre09.g415500		*petA* translation initiation

Translation of *petB* (from its monocistronic transcript) and *petD* (from the dicistronic *petB–petD* transcript) occurs on free, stromal polysomes, suggesting that these two subunits are post-translationally inserted into the thylakoid membrane (Friemann and Hachtel, 1988). Thylakoid membrane insertion of both PetB and PetD seems to be supported by the Sec translocase (Zak *et al.*, 1997). After its insertion into the thylakoid membrane, apocytochrome b_6 binds its two *b*-type haems: the non-covalent binding of the low potential haem at the luminal side is a prerequisite for the addition of the high potential haem at the stromal side (Kuras *et al.*, 1997; Dreher *et al.*, 2008). Finally, haem c_i is covalently attached (Saint-Marcoux *et al.*, 2009). At least *in vitro*, the binding of both haems *b* to apocytochrome b_6 is spontaneous and occurs in the absence of auxiliary proteins (Dreher *et al.*, 2008). This implicates the presence of free haems in the thylakoid membrane, even though they could act as photosensitizers and might cause oxidative damage to the photosynthetic apparatus (Nakamoto *et al.*, 2000). The subsequent binding of haem c_i via an unusual single thioether bond with a conserved cysteine residue involves a complex enzymatic insertion system called 'system IV' or the CCB system (for '*c*ofactor assembly on complex *C* subunit *B*').

So far, four proteins in this CCB system have been identified and characterized both in *C. reinhardtii* and in *A. thaliana* (Kuras *et al.*, 2007; Lyska *et al.*, 2007; Lezhneva *et al.*, 2008; Saint-Marcoux *et al.*, 2009; reviewed by de Vitry, 2011). All CCB proteins are transmembrane proteins in the thylakoids, but, astonishingly, none of these proteins contains an obvious haem-binding site. The first step of haem c_i insertion is the formation of a subcomplex between apocytochrome b_6 and CCB1. Then apocytochrome b_6 is transferred from CCB1 to CCB3, which next recruits a CCB2–CCB4 heterodimer to form a large CCB2–CCB3–CCB4–cytochrome b_6 complex, in which the ligation reaction between the apoprotein and haem c_i seems to occur. After that, the mature cytochrome b_6 may associate with subunit IV.

In higher plants, *petA* is the final gene of a large polycistronic unit also comprising the small PSI subunit gene *psaI*, the PSI assembly factor *ycf4* (Krech *et al.*, 2012), and the open reading frame *ycf10*. Both the tetracistronic transcript and smaller processed versions of the cistron, especially the monocistronic *petA* transcript, are translated by thylakoid-bound polysomes (Friemann and Hachtel, 1988). The N-terminal pre-sequence of the nascent PetA polypeptide is essential for correct thylakoid insertion of cytochrome *f* (Zak *et al.*, 1997). It targets the ribosomes to the thylakoids, where PetA is co-translationally inserted into the membrane through the Sec translocase (Voelker and Barkan, 1995; Röhl and van Wijk, 2001). After its membrane insertion, the N-terminal pre-sequence facing towards the thylakoid lumen is cleaved off by the thylakoid processing peptidase. Then, the covalently bound haem is inserted via a 'CCS factor' or 'system II' mechanism, with 'CCS' standing for '*c*-type *c*ytochrome *s*ynthesis' system. This system has been characterized in much more detail than the CCB system, not least because it is conserved for all *c*-type cytochromes of the bc_1 complexes, and also functions in the maturation of the luminal redox carrier cytochrome *c* in algae (reviewed by Nakamoto *et al.*, 2000; Karamoko *et al.*, 2013).

The CCS system can be split into two components: a system for haem transfer from the stromal side of the thylakoid membrane to the lumen and its ligation to the apocytochrome *f* (see below), and a disulphide-reducing system, which is required to keep the two cysteines of apocytochrome *f* in their reduced state, so that they can form the thioether bonds with the vinyl groups of the haem. Because no reducing power can be directly generated in the thylakoid lumen, electrons need to be transferred from the stromal side of the thylakoid membrane to the thylakoid lumen. For that reaction, the stromal thioredoxin *m* functions as electron donor (Motohashi and Hisabori, 2006, 2010). In *C. reinhardtii*, thioredoxin *m* transfers its electrons via the thylakoid-intrinsic thiol disulphide transporter protein CCDA (Page *et al.*, 2004; Motohashi and Hisabori, 2010) towards the CCS5 protein (Gabilly *et al.*, 2011). HCF164 is the CCS5 homologue in higher plants (Lennartz *et al.*, 2001; Motohashi and Hisabori, 2006). HCF164 is a membrane-intrinsic thioredoxin-like protein with a large luminal domain, which has a disulphide reductase activity towards apocytochrome *f*. In *C. reinhardtii*, the CCS4 protein has been identified as an additional component of the disulphide-reducing system, but its precise function remains to be determined (Gabilly *et al.*, 2011). Because CCS4 does not have a classical N-terminal signal peptide and has not been identified in proteomics analysis of the thylakoid membrane, its subcellular localization is unclear. However, because the defect of the disulphide-reducing system in a *ccs4* mutant can be partly rescued either by overexpressing the thiol disulphide transporter CCDA or by the addition of thiols such as dithiothreitol (DTT), it is speculated that CCS4 functions either together or in parallel with CCDA in the transfer of electrons from the stromal to the luminal side of the thylakoid membrane (Gabilly *et al.*, 2011). CCS4 has a limited structural similarity to the *A. thaliana* HCF153 protein (Karamoko *et al.*, 2013). The HCF153 mutant of *A. thaliana* suffers from reduced accumulation of the cytochrome b_6f complex, but there too the precise mechanistic basis of the cytochrome b_6f complex accumulation defect remains unknown (Lennartz *et al.*, 2006). In addition to providing electrons for the reduction of apocytochrome *f*, the disulphide-reducing system constituted by thioredoxin *m*, CCDA, and possibly HCF153 may also play a role in the reduction of several other thylakoid lumen proteins, including the Rieske protein and the small luminal PSI subunit PsaN, but the functional significance of this reduction remains to be elucidated (Motohashi and Hisabori, 2006; Karamoko *et al.*, 2013).

Because the ferrochelatase catalysing the final step of haem biosynthesis is attached to the stromal side of the thylakoid membrane, a system for the controlled delivery of the newly formed haem to the reduced thiol side chains of the luminal domain of apocytochrome *f* is required. So far, two components of the haem transfer and ligation system have been identified: the plastid-encoded CcsA protein (Xie and Merchant, 1996) and the nuclear-encoded CCS1 protein (Inoue *et al.*, 1997). Both are thylakoid-intrinsic proteins,

and the accumulation of CCS1 is severely impaired in a *ccsA* mutant strain and in mutants affected in the nuclear-encoded CCS components, possibly indicating a close interaction of all CCS proteins, and the formation of a large 'CCS complex' involved in haem ligation to apocytochrome *f* (Dreyfuss *et al.*, 2003). CcsA has a haem-binding domain on the luminal side and may be the direct haem donor for the ligation into apocytochrome *f*.

A surprising feature of cytochrome *f* is that in the absence of its assembly partners, it is relatively stable in some species, while it is rapidly degraded in others. For example, in an *A. thaliana* PETC knock-out mutant, which does not accumulate functional cytochrome b_6f complexes, free cytochrome *f* can still be detected, while all other subunits of the cytochrome b_6f complex are rapidly degraded (Maiwald *et al.*, 2003). In *C. reinhardtii*, mutants deficient in the plastome-encoded subunits cytochrome b_6 and subunit IV also accumulate detectable amounts of cytochrome *f*, while in *Lemna perpusilla* and tobacco free cytochrome *f* is rapidly degraded in the absence of one of the other essential subunits of the complex (Bruce and Malkin, 1991; Monde *et al.*, 2000; Hojka *et al.*, 2014). The basis of this differential behaviour of cytochrome *f* in different species is still unknown.

The nuclear-encoded Rieske protein (PETC) is translated by cytosolic ribosomes as a precursor protein with a long N-terminal chloroplast transit peptide, which after import into the chloroplast is removed by the stromal processing peptidase (Fig. 3). Once in the stroma, the fate of the Rieske protein differs from that of almost all other nuclear-encoded, membrane-intrinsic and luminal proteins, in that its transfer to the thylakoid membrane is extremely slow (Madueno *et al.*, 1993; Molik *et al.*, 2001). A significant fraction of newly imported Rieske protein first interacts with the chloroplast Cpn60 chaperonin system (Madueno *et al.*, 1993) and possibly the cpSRP54 protein (High *et al.*, 1997). Then, even though the Rieske protein does not have a typical N-terminal TAT sequence, its membrane insertion is mediated by the TAT pathway, which is normally involved in the translocation of fully folded proteins into the thylakoid lumen (Molik *et al.*, 2001). The complete hydrophobic N-terminal domain of the Rieske protein, which forms a transmembrane helix in the thylakoid membrane, seems to function as a signal domain for recognition by the TAT pathway. The precise time point of the 2Fe2S cluster insertion into the Rieske protein is still enigmatic. The biogenesis of 2Fe2S clusters occurs in the stroma, and all other chloroplast proteins harbouring 2Fe2S clusters are localized there as well (reviewed by Ye *et al.*, 2006). Therefore, it seems likely that the attachment of the 2Fe2S cluster to the Rieske apoprotein occurs in the stroma. This could also explain why the Rieske protein uses the TAT pathway for membrane insertion, which transports fully folded proteins. However, mutated versions of the Rieske apoprotein unable to bind their 2Fe2S cluster can still be inserted into the thylakoid membrane and allow the stable accumulation of the cytochrome b_6f complex in pea (Kapazoglou *et al.*, 2000). Therefore, it is still a matter of debate if the 2Fe2S cluster is already attached to the apoprotein in the stroma, or if its attachment occurs by a so far unknown pathway in the thylakoid lumen.

In addition to the auxiliary proteins involved in haem attachment to cytochromes b_6 and *f*, DAC (defective in the accumulation of the cytochrome b_6f complex), also named CPLD38 (conserved in the Plantae and diatoms), has been identified as an auxiliary protein required for cytochrome b_6f complex biogenesis both in *C. reinhardtii* (Heinnickel *et al.* 2013) and in higher plants (Xiao *et al.*, 2012). In *A. thaliana*, it may be involved in PetD insertion into the cytochrome b_6f complex (Xiao *et al.*, 2012).

Regulation of cytochrome b_6f *complex accumulation*

It is still unclear which steps of cytochrome b_6f complex biogenesis ultimately control its accumulation. Based on the assumption that cytochrome b_6f complex assembly begins with cytochrome b_6 and ends with the insertion of the Rieske protein or one of the small peripheral subunits, a regulation at the level of cytochrome b_6 synthesis appears to be most likely. The regulation of cytochrome b_6f complex biogenesis has been extensively studied in the green alga *C. reinhardtii*, where the stoichiometric accumulation of the large chloroplast-encoded subunits is ensured by a complex process of translational autoregulation, called 'Control by Epistasy of Synthesis' (CES). Translation of the *petA* mRNA is strongly down-regulated whenever free PetA protein accumulates, but it is stimulated when PetA assembles with the other subunits (Kuras and Wollman, 1994). This negative feedback regulation of free cytochrome *f* on its own translation is mediated by a five amino acid motif at its C-terminus (Choquet *et al.*, 2003), which determines the stability of the PPR protein MCA1, a nuclear-encoded factor controlling the accumulation of *petA* mRNA and enhancing its translation (Loiselay *et al.*, 2008; Boulouis *et al.*, 2011). In the presence of unassembled cytochrome *f*, the exposed C-terminal motif of PetA targets MCA1 for proteolytic degradation, preventing it from stabilizing the *petA* mRNA. Upon assembly of PetA into the cytochrome b_6f complex and the resulting concealment of the regulatory motif, MCA1 accumulates and can interact with the 5'-untranslated region (UTR) of the *petA* transcript, allowing its stabilization and activation of its translation (Boulouis *et al.*, 2011). MCA1 possesses several features that make it a good regulatory protein: it is short lived, and its accumulation varies under different physiological conditions (Raynaud *et al.*, 2007). Thus, in *C. reinhardtii*, the biogenesis of the cytochrome b_6f complex is limited both by the availability of cytochrome b_6 and subunit IV for complex assembly of PetA, and by the MCA1-controlled cytochrome *f* synthesis.

It is unknown if a similar CES mechanism exists in higher plants. The C-terminal regulatory motif of PetA is highly conserved in all photosynthetic eukaryotes, and in transplastomic tobacco knock-out plants of *petB* and *petD*, polysome association of the *petA* mRNA is somewhat reduced (Monde *et al.*, 2000). However, neither MCA1 nor TCA1, a pioneer nuclear-encoded protein activating the translation of the *petA* mRNA in *C. reinhardtii* (Raynaud *et al.*, 2007), possess homologues in higher plants. The mechanism underlying the

decreased *petA* mRNA translation in tobacco remains to be elucidated.

In higher plants, the PRFB3 protein stabilizing the 3' region of the *petB* transcript decreases strongly in dim light, and therefore has been postulated to control changes in cytochrome b_6f complex accumulation in response to different light intensities (Stoppel *et al.*, 2011; see also 'Light and redox signals' above). However, because in different mutant lines only a repression of the PFRB3 protein to <25% of wild-type levels clearly affected the accumulation of the cytochrome b_6f complex (Stoppel *et al.*, 2011), it appears unlikely that PRFB3 is a major regulator of cytochrome b_6f complex accumulation. Furthermore, a regulation on the level of mRNA abundance of the structural subunits can certainly be excluded for the leaf age-dependent repression of the *de novo* biogenesis of the cytochrome b_6f complex in mature leaves of tobacco (Schöttler *et al.*, 2007; Hojka *et al.*, 2014; for details, see 'Degradation of chloroplast ATP synthase and the cytochrome b_6f complex'). The expression of both nuclear-encoded subunits of the cytochrome b_6f complex decreases slowly with leaf age, and the expression of the three small plastid-encoded subunits is also only weakly reduced in mature leaves. For the *petA*, *petB*, and *petD* transcripts, no significant leaf age-dependent differences in mRNA accumulation could be observed at all (Hojka *et al.*, 2014). Most probably, the loss of *de novo* biogenesis of the cytochrome b_6f complex is attributable to the shut-down of mRNA translation via the repression of nuclear-encoded, so far unidentified, specific translation initiation factors.

In *A. thaliana*, import of the precursor of the Rieske protein into the chloroplast was suggested to be negatively regulated by the precursor of the thylakoid lumen immunophilin AtFKBP13, because AtFKBP13 RNAi lines accumulated increased amounts of mature Rieske protein in the thylakoid membrane (Gupta *et al.*, 2002). However, in another investigation of AtFKBP13 function, no changes in the abundance of the Rieske protein itself or the other cytochrome b_6f complex subunits were observed (Ingelsson *et al.*, 2009). It seems possible that the CCB pathway controls cytochrome b_6f complex biogenesis, because it is specific for haem c_i ligation to cytochrome b_6, while the CCS pathway also acts in the maturation of other proteins, such as the soluble redox carrier cytochrome c_6 in *C. reinhardtii* (Kuras *et al.*, 2007; Lyska *et al.*, 2007; Lehzneva *et al.*, 2008; Gabilly *et al.*, 2011). A limiting function of DAC also cannot be excluded. At least for PSI accumulation, a limiting function of auxiliary proteins involved in its assembly has been demonstrated (reviewed by Schöttler *et al.*, 2011). However, a control of cytochrome b_6f complex biogenesis at the late stages of cofactor synthesis or complex assembly would be a rather inefficient and wasteful process, and therefore seems less likely.

Biogenesis of chloroplast ATP synthase

The biogenesis of chloroplast ATP synthase has to be tightly controlled to avoid futile reaction cycles either dissipating the pmf across the thylakoid membrane or consuming ATP in the stroma. In bacteria, CF_1 assembly intermediates containing the $\alpha_3\beta_3$ catalytic centre have a significant ATPase activity (Koebmann *et al.*, 2002); therefore, their accumulation has to be avoided, or they have to be kept in an inactive state, possibly by interactions with auxiliary proteins. Also the assembly of a complete CF_0 subcomplex in the thylakoid membrane may be detrimental, because, at least in *Escherichia coli*, free F_0 can function as a membrane uncoupler (Birkenhäger *et al.*, 1999; Franklin *et al.*, 2004). In a similar way, the presence of CF_0 subcomplexes with an open proton channel may result in the uncontrolled dissipation of the pmf across the thylakoid membrane. Therefore, chloroplast ATP synthase assembly has to proceed in such a way that the formation or at least the accumulation of a functional H^+-translocating subcomplex of CF_0 is avoided. This introduces a new level of complexity in the control and co-ordination of subunit biosynthesis. A list of all auxiliary proteins involved in the biogenesis of ATP synthase is provided in Table 4.

Subunit synthesis and assembly

The three nuclear-encoded ATP synthase subunits, ATPC, ATPD, and ATPG, are translated on cytosolic ribosomes and

Table 4. *List of auxiliary proteins involved in the biogenesis of chloroplast ATP synthase*

Information is provided on the protein name, whether it is encoded in the nucleus (N) or chloroplast (C), the gene locus number in both *A. thaliana* and *C. reinhardtii*, alternative names, and gene function.

Name	Gene locus	Alternative name/orthologues	Function
ALB4 (N)	AT1G24490	–	Assembly/stability of the ATP synthase
ATP1 (N)	Locus not identified		Translation of *atpB*
CGL160 (N)	AT2G31040	–	Assembly of the ATP synthase
MDA1 (N)	Only in *C. reinhardtii*: locus not identified		*atpA* mRNA stability
PPR10 (N)	Only in higher plants: At2g18940		PPR protein involved in *atpH* transcript stabilization and translation initiation
SVR7 (N)	Only in higher plants: AT4G16390	ATP4, P67	PPR protein involved in translation initiation of *atpA*, *atpB/E* mRNA, accumulation of *atpF* mRNA
TDA1 (N)	Only in *C. reinhardtii*: Cre08.g358350		Octotrico peptide repeat protein, functions in translation initiation of *atpA*

post-translationally imported into the chloroplast. The CF_1 subunits ATPC and ATPD undergo N-terminal processing of their chloroplast transit peptides by the stromal processing peptidase to generate the mature protein, possibly interact with stromal chaperones, and then are incorporated into CF_1. The CF_0 subunit ATPG (subunit b') differs from ATPC and ATPD in that its pre-sequence is considerably longer and has a bipartite structure consisting of a chloroplast transit peptide and a second targeting sequence similar to those of luminal proteins, which mediates thylakoid membrane insertion of ATPG. This bipartite pre-sequence lacks an intermediate cleavage site for the stromal processing peptidase, so that the complete pre-sequence is retained until ATPG is inserted into the thylakoid membrane by the spontaneous pathway, which is independent of the pmf across the thylakoid membrane, of nucleotides, and of stromal factors (Michl *et al.*, 1994). Only after membrane insertion is the N-terminal bipartite pre-sequence facing towards the thylakoid lumen cleaved off by the luminal thylakoid processing peptidase (Michl *et al.*, 1994).

In vascular plants, the plastome-encoded subunits of ATP synthase are organized in two polycistronic units. The smaller polycistronic unit comprises *atpB* and *atpE*, whose coding regions partially overlap. The larger co-transcriptional unit comprises the ribosomal protein gene *rps2*, followed by *atpI* (the a-subunit of CF_0), *atpH* (the c-subunit of CF_0), *atpF* (the CF_0 subunit b), and *atpA* (the α-subunit of CF_1). In spinach, a second promoter located between the coding regions of *atpI* and *atpH* allows the expression of a shorter tricistronic transcript only covering *atpH*, *atpF*, and *atpA* (Hotchkiss and Hollingsworth, 1997). It has been suggested that these two transcription start sites, together with extensive RNA cleavage and processing, allow the chloroplast to produce the different mRNAs in abundances which closely match the protein stoichiometry later needed for ATP synthase assembly. In particular, the *atpH* transcript coding for the 14 c-ring subunits is much more abundant than any of the other transcripts (Hotchkiss and Hollingsworth, 1997). Specific translation initiation factors exist at least for some of the open reading frames. In maize, after mRNA cleavage in the intergenic region between *atpI* and *atpH*, the PPR10 protein binds to the 5'-UTR of *atpH* and protects it against exonucleases (Pfalz *et al.*, 2009). This special protection of its 5'-UTR may also explain the much higher abundance of the *atpH* transcript relative to all other ATP synthase genes encoded by the polycistronic unit, which was observed in spinach (Hotchkiss and Hollingsworth, 1997; see above). Additionally, PPR10 strongly enhances translation initiation of the *atpH* open reading frame, either by maintaining the ribosome-binding site of *atpH* in an accessible conformation or via a direct interaction with the ribosome (Pfalz *et al.*, 2009; Zoschke *et al.*, 2013*a*). Furthermore, the *atpB* translation initiation factor ATP4 (see below) also enhances translation of the *atpA* open reading frame (Zoschke *et al.*, 2012, 2013*b*).

The mRNAs of the large co-transcriptional unit are translated on free polysomes in the stroma, suggesting that the membrane insertion of newly translated CF_0 subunits occurs as a post-translational process (Friemann and Hachtel, 1988; see below). Despite the fact that the plastid-encoded b-subunit (AtpF) is highly homologous to the nuclear-encoded b'-subunit (ATPG), which inserts spontaneously into the thylakoid membrane, the membrane insertion of AtpF is dependent on the Sec translocase (Michl *et al.*, 1999). After thylakoid insertion, a short N-terminal sequence of subunit b is cleaved off, yielding the mature b-subunit, which then assembles into the complex (see below). The function of the cleavable N-terminal peptide of AtpF is not known, because it is dispensable for thylakoid membrane insertion (Michl *et al.*, 1999).

In the case of the *atpB*/*atpE* co-transcriptional unit, in maize, the so-far unidentified nuclear-encoded ATP1 protein plays a specific role in translation initiation of the *atpB* open reading frame (McCormac and Barkan, 1999; Zoschke *et al.*, 2013*a*). Additionally, the PPR protein ATP4 strongly promotes translation of *atpB* (Zoschke *et al.*, 2012). A similar function has been shown for its homologue SVR7 in *A. thaliana* (Zoschke *et al.*, 2013*b*). *atpB* and *atpE* are translated by free polysomes, in line with their stromal localization (Friemann and Hachtel, 1988).

Meaningful insights into the assembly of chloroplast ATP synthase can be obtained by the comparison with the much better understood assembly process of F_0F_1 ATP synthases in prokaryotes, especially in *E. coli*. While the structure of the F_1 moiety is highly conserved in all organisms, the structure of F_0 differs greatly. Both the *E. coli* ATP synthase and the mitochondrial ATP synthase of yeast contain 10 instead of the 14 c-subunits found in chloroplast ATP synthase (reviewed by von Ballmoos *et al.*, 2008). Also, prokaryotes contain two b-subunits in their F_0 moiety, which are very similar to b and b' in chloroplasts. In mitochondria, the subunit composition of F_0 can be much more complex than in bacteria and chloroplasts. Therefore, F_0 assembly in *E. coli* is a much better model for chloroplast ATP synthase biogenesis than ATP synthase assembly in yeast mitochondria.

Assembly of the c_{10}-ring in *E. coli* begins with the post-translational membrane insertion of the individual c-subunits, which in *E. coli* is strictly dependent on the protein membrane insertase YidC (Yi *et al.*, 2003; van der Laan *et al.*, 2004; Robinson and Woolhead, 2013). YidC may also facilitate the ring formation of the c-subunits (Yi *et al.*, 2003). In the chloroplast, two homologues of YidC exist: ALB3 functions as the major thylakoid membrane insertase and, in a split-ubiquitin system, it has been shown to interact with the c-subunit of CF_0 (Pasch *et al.*, 2005). ALB4 is the second chloroplast homologue of YidC. ALB4 does not function as a general thylakoid membrane insertase, but instead acts as a specific auxiliary protein for ATP synthase assembly, in that it interacts with both CF_1 and CF_0 subunits and possibly plays a role in the attachment of both ATP synthase moieties to each other, instead of only facilitating the assembly of the c-ring structure (Benz *et al.*, 2009).

In addition to YidC, the UncI protein also acts as a non-essential chaperone supporting c-ring formation (Suzuki *et al.*, 2007). The UncI homologue of chloroplasts, CGL160 (for: Conserved only in the Green Lineage 160), physically

interacts with the c-subunit of CF_0 and may stabilize the c-ring (Rühle *et al.*, 2014). Similar to the role of UncI in most bacteria, CGL160 is not essential for ATP synthase accumulation: in an *A. thaliana* T-DNA insertion line completely deficient in CGL160 protein, still ~20% of the wild-type level of ATP synthase accumulates (Rühle *et al.*, 2014).

Both in *E. coli* and in mitochondria, the assembled c-ring interacts with an F_1-subcomplex consisting of $\alpha_3\beta_3\gamma\varepsilon$, which is first formed in the stroma (Rak *et al.*, 2011; Deckers-Hebestreit, 2013). Not much is known about the assembly of this $\alpha_3\beta_3\gamma\varepsilon$ subcomplex. The nucleotide binding domains on the α- and β-subunit are hydrophobic and in the fully assembled CF_1 complex protected by their binding to the neighbouring subunit; however, directly after their translation, they may require specific chaperones to prevent these hydrophobic domains from unspecific aggregation. In the chloroplast, free α- and β-subunits interact with different chaperones, and a mixture of stromal chaperones can reconstitute active CF_1 from denatured subunits (Chen and Jagendorf, 1994). The presence of Cpn60 in the chaperone mixture is essential to obtain reconstitution. Cpn60 binds free α-, β-, and γ-subunits of CF_1 (Chen and Jagendorf, 1994). However, because the purified stromal chaperone Cpn60 alone is not sufficient to allow the *in vitro* reconstitution of ATPase activity of CF_1, so far non-identified co-chaperones co-operating with Cpn60 seem to be essential for CF_1 assembly in chloroplasts. In *C. reinhardtii*, at least $\alpha_3\beta_3$ and $\alpha_3\beta_3\gamma$ subcomplexes have been found, further supporting an assembly pathway of CF_1 similar to that identified in *E. coli* (Drapier *et al.*, 2007).

After the interaction between the c-ring and the $\alpha_3\beta_3\gamma\varepsilon$ CF_1 subcomplex has been established, this subcomplex seems to interact weakly with an ab_2 subcomplex in both *E. coli* (Deckers-Hebestreit, 2013) and yeast (Rak *et al.*, 2011). Ultimately, the interaction of the ab_2 subcomplex and $\alpha_3\beta_3\gamma\varepsilon c_{10}$ is stabilized by the binding of the last F_1-subunit δ to the assembly intermediate, resulting in the formation of the mature ATP synthase holoenzyme (Hilbers *et al.*, 2013). Thus, only after the stabilization of the ATP synthase holoenzyme by addition of the δ-subunit of F_1 is a complex capable of proton translocation and ATP synthesis established, and a futile dissipation of the pmf by assembly intermediates is effectively prevented. However, once a stable association of ab_2c_{10} has been established, F_0 remains active as a proton channel even after F_1 has been dissociated from it, indicating that the δ-subunit only helps ab_2 to adopt a structure which then allows a strong and stable interaction with c_{10} (Deckers-Hebestreit, 2013).

It is still unknown if these later assembly steps occur in a similar way in chloroplasts. Data supporting a similar assembly pathway of chloroplast ATP synthase were obtained in *C. reinhardtii* (Lemaire and Wollman, 1989): while mutants deficient in CF_1 subunits do not accumulate CF_0 subunits due to their rapid degradation, in the absence of different CF_0 proteins, CF_1 subunits can still be detected in the stroma. While the knock-out of the c-ring completely abolishes membrane association of CF_1, subunit IV (a) is not strictly required for their membrane association. A mutant deficient in subunit I (b) displays an intermediate defect in CF_1 accumulation and association with the thylakoid membrane. This is in line with the *E. coli* scenario where assembly of $\alpha_3\beta_3\gamma$ with the c-ring mediates the membrane association of CF_1 subunits and precedes binding of that subcomplex to the peripheral stalk, and where association of the a-subunit is among the latest steps of ATP synthase assembly.

In comparison with the number of auxiliary proteins, which support the biogenesis of the other photosynthetic complexes, surprisingly few auxiliary proteins involved in chloroplast ATP synthase assembly have been identified to date. It is possible that due to the absence of redox-active cofactors in ATP synthase, a much lower number of auxiliary proteins is needed for its assembly, because in the case of the cytochrome b_6f complex, the vast majority of auxiliary proteins are involved in haem insertion into the complex (see 'Subunit synthesis and cofactor attachment'). Several auxiliary proteins involved in ATP synthase biogenesis have been identified in *E. coli* and especially in yeast mitochondria (Rak *et al.*, 2011; Deckers-Hebestreit, 2013), but many of these do not have obvious homologues in chloroplasts. Based on existing evidence, in the chloroplast at least, the existence of additional chaperones involved in CF_1 assembly has been predicted (Chen and Jagendorf, 1994). In a Mu transposon library in maize, two more ATP synthase-deficient mutants called ATP2 and ATP3 have been isolated but, because the affected nuclear-encoded proteins have not been identified until now, it is still unclear if these two proteins function in the gene expression and translation of ATP synthase subunits, similarly to ATP1 and ATP4 (see above), or if they play a role in ATP synthase assembly (Zoschke *et al.*, 2012).

The identification of non-essential auxiliary proteins involved in ATP synthase biogenesis may be more difficult than in case of all other photosynthetic complexes, because only a >50% reduction in ATP synthase abundance results in a clear photosynthetic defect, at least under stable growth conditions in the laboratory (Rott *et al.*, 2011; see 'chloroplast ATP synthase'). Therefore, mutants affected in non-essential auxiliary proteins involved in ATP synthase biogenesis such as CGL160 do not display a severe phenotype under controlled growth conditions and may remain unnoticed in mutant screens, because their photosynthetic defects are too small. Therefore, a sensitive and saturating screen for auxiliary factors required for ATP synthase biogenesis may be difficult to achieve.

Regulation of ATP synthase accumulation

The regulation of ATP synthase biogenesis has been studied in most detail in *C. reinhardtii*. The organization of ATP synthase subunits in this green alga differs dramatically from that found in higher plants, as all plastid-encoded ATP synthase subunit genes are dispersed over the plastome and form co-transcriptional units together with genes of other complexes (Woessner *et al.*, 1987). Also, several nuclear-encoded proteins involved in the expression and translation of plastid-encoded ATP synthase subunits do not have obvious homologues in higher plants (see below). Therefore, it is not clear

to what extent results obtained in *C. reinhardtii* are valid for higher plants.

Transcript abundance at least of the *atpA* mRNA is unlikely to have a limiting effect on ATP synthase accumulation in *C. reinhardtii*: in a mutant suffering from a reduction in *atpA* mRNA abundance to <10% of wild-type levels, ATP synthase still accumulates to ~50% of wild-type levels (Drapier *et al.*, 2002). Instead, the main level of regulation is translation initiation: the nuclear-encoded TDA1 protein (*T*ranslation of complex *D* α-subunit 1) is a specific activator of *atpA* mRNA translation in *C. reinhardtii*. TDA1 is an octotrico peptide repeat protein, which activates *atpA* mRNA translation via interaction with the 5'-UTR of *atpA* mRNA (Eberhard *et al.*, 2011). A second nuclear-encoded factor, whose mutation completely abolishes *atpB* transcript accumulation, has not been identified to date (Drapier *et al.*, 1992).

Similar to the cytochrome b_6f complex, for the assembly of CF_1 a CES process also ensures the stoichiometric production of subunits in *C. reinhardtii* (Drapier *et al.*, 2007): AtpB is only synthesized when the newly translated protein is rapidly assembled into intact CF_1 or subcomplexes at least consisting of $\alpha_3\beta_3\gamma$. To ensure that sufficient amounts of AtpA are produced for assembly, free AtpB promotes the translation of the *atpA* mRNA. However, when αβ dimers or $\alpha_3\beta_3$ hexamers accumulate in the stroma without further assembling into the holoenzyme, because availability of the nuclear-encoded γ-subunit is limiting, translation of the *atpB* mRNAs is strongly repressed. Because AtpB protein accumulation drops in response to this repression, translation of the *atpA* mRNA is also no longer stimulated. Only after assembly of the $\alpha_3\beta_3$ hexamer proceeds by association with the γ-subunit is the feedback inhibition of $\alpha_3\beta_3$ on the translation of *atpB* mRNA attenuated. These data suggest that at least for the synthesis of CF_1 and its subcomplexes, the availability of the nuclear-encoded γ-subunit may be rate limiting (Drapier *et al.*, 2007). It is still unknown if direct interactions between the free AtpB subunit and the *atpA* translation activator TDA1 occur, which would then switch TDA1 to a translational activation mode (Eberhard *et al.*, 2011).

Studies of AtpB translation mutants in maize argue against a similar CES mechanism in higher plants: in the ATP1 mutant, which suffers from a massive defect in *atpB* translation, translation of *atpA* is almost unaffected (McCormac and Barkan, 1999; Zoschke *et al.*, 2013*a*). However, a rate-limiting function for ATPC in ATP synthase biogenesis in vascular plants and for *atpB* translation in the chloroplast is tentatively supported by studies using *ATPC* antisense plants in tobacco, and transplastomic *atpB* translation initiation mutants (Rott *et al.*, 2011). Interestingly, translation of *ATPC* mRNA is stimulated by cytokinin application (Sherameti *et al.*, 2004).

Photosynthetic complex lifetimes and turnover

Our knowledge on the lifetimes of most photosynthetic complexes and their turnover was rather limited until very recently. Only the turnover of PSII has been studied for a long time, because the plastome-encoded PSII reaction centre subunit D1 (PsbA) is highly prone to oxidative damage. Depending on the light intensity, turnover rates of the D1 protein varying between once per day and once per hour have been reported (Aro *et al.*, 1993; He and Chow, 2003). Instead of a full degradation of the damaged PSII, only the damaged D1 protein is replaced by a specific PSII repair cycle. Only under light stress conditions, when the rate of damage of the PSII reaction centre exceeds the capacity of the repair cycle, do damaged PSII reaction centres start to accumulate in the thylakoids (Aro *et al.*, 2005; Rokka *et al.*, 2005).

The other photosynthetic complexes may be much more stable than PSII. Therefore, they may not have comparable repair cycles. In the green alga *C. reinhardtii*, inhibition of chloroplast translation results in rapid loss of PSII, while the decay of the other photosynthetic complexes is much slower. For the cytochrome b_6f complex, a half-life well above 21 h was calculated (Gong *et al.*, 2001). However, the accumulation of damaged PSII might impair the general stability of the photosynthetic apparatus: due to an increased production of ROS, the lifetime of the cytochrome b_6f complex might have been substantially underestimated.

In tobacco, loss of the small peripheral L subunit of the cytochrome b_6f complex does not alter complex accumulation in young, expanding leaves, but it strongly destabilizes the whole complex and accelerates the leaf age-dependent loss of the cytochrome b_6f complex in mature leaves (Schöttler *et al.*, 2007). Presumably, young leaves compensate for the reduced stability in the Δ*petL* transformant by an increased *de novo* biogenesis of the cytochrome b_6f complex. In older leaves, the reduced complex stability can no longer be compensated by *de novo* synthesis. This suggests a strong ontogenetic repression of cytochrome b_6f complex biogenesis in fully expanded leaves. Assuming that cytochrome b_6f complex biogenesis is completely switched off in mature tobacco leaves, the slow decline in cytochrome b_6f complex contents with increasing leaf age in wild-type tobacco would directly reflect its half-life, which would be in the range of ~1 week.

To determine directly the lifetime of the cytochrome b_6f complex, an ethanol-inducible RNAi approach against the two essential nuclear-encoded subunits PETM and PETC was used (Hojka *et al.*, 2014). RNAi induction in young leaves still assembling their photosynthetic apparatus resulted in a drastic repression of cytochrome b_6f complex accumulation. However, in mature leaves, whose photosynthetic apparatus was already fully established prior to RNAi induction, no differences in cytochrome b_6f complex contents between the wild type and the induced transformants were observed, even after 14 d of induction. In both the wild type and the induced RNAi plants, the cytochrome b_6f complex content decreased by ~50% per week. Because only the two nuclear-encoded subunits were targeted by the inducible RNAi approach, it cannot be excluded that some plastid-encoded subunits have a higher turnover than the two nuclear-encoded subunits. This would require a scenario where, upon damage, an unstable plastid-encoded subunit would be rapidly replaced, while the more stable nuclear-encoded subunits would have to be

reincorporated into the repaired cytochrome b_6f complex. This appears to be unlikely, because a comparison of radiolabel incorporation into photosynthetic protein complexes in young and old bean leaves showed that while in young leaves labelling of all photosynthetic complexes was easily detectable, in old leaves, labelling of cytochrome b_6f complex subunits no longer occurred. Radiolabelling of plastid-encoded subunits of the ATP synthase and PSI was also strongly decreased in old leaves, and only labelling of PSII reaction centre subunits was detectable (Roberts *et al.*, 1987). This is compatible with a high stability of the cytochrome b_6f complex and a restriction of its biogenesis to young leaves still establishing their photosynthetic apparatus. It further suggests that PSI and ATP synthase might also be highly stable, at least when plants are grown under non-stressed conditions.

Recent non-targeted proteomics studies in *A. thaliana* and barley suggest that a high stability may be a general feature of bioenergetic complexes in mitochondria and chloroplasts (Nelson *et al.*, 2013, 2014): complex turnover was analysed after the addition of ^{15}N to label newly synthesized proteins. Protein degradation was followed as the decrease of unlabelled protein synthesized before the addition of ^{15}N, relative to the total pool of labelled and unlabelled protein. PetA, PetB, and PETC were detected in this analysis, and all displayed degradation rates of ~0.1 d^{-1} or even less, well in line with the very low turnover rate of the cytochrome b_6f complex suggested by the targeted repression of PETC and PETM in the inducible tobacco RNAi plants (Hojka *et al.* 2014). A similar stability was observed for complex III of the respiratory electron transport chain in *A. thaliana* mitochondria (Nelson *et al.*, 2013).

Furthermore, the barley proteome analysis of protein turnover supports a very high stability of PSI and ATP synthase, in that the average degradation rate of ATP synthase proteins is only 0.1 d^{-1}, and that of PSI subunits is even lower (Nelson *et al.*, 2014). Even most PSII subunits, with the exception of the D1 protein, are highly stable. A high stability of PSI is also supported by the observation that two auxiliary proteins required for its assembly, Y3IP1 and Ycf4, only accumulate in young tobacco leaves (Krech *et al.*, 2012), suggesting that the biogenesis of PSI might be restricted to young leaves in a similar way to that of the cytochrome b_6f complex. Similarly, the ontogenetic loss of ATP synthase content with increasing leaf age in tobacco might be due to a restriction of ATP synthase biogenesis to young leaves, and then the ontogenetic decrease would simply reflect the degradation rate of ATP synthase.

This relatively high stability and slow turnover of photosynthetic complexes may partly explain why the capacity of leaves of annual plants to adjust to environmental perturbations decreases with increasing leaf age: in tobacco, only young, still expanding leaves show the full capacity to acclimate to changing environmental conditions, while fully developed leaves only have a limited capacity for acclimation, for example to changes in actinic light intensity (Hojka *et al.*, 2014). This is probably due to the developmental repression of complex biogenesis in leaves once their photosynthetic apparatus has been fully established. Additionally, morphological constraints may limit the acclimation capacity of mature leaves, because mature leaves cannot increase stomatal density or vein density to increase CO_2 uptake and photoassimilate export.

Degradation of chloroplast ATP synthase and the cytochrome b_6f complex

Under certain environmental conditions such as drought stress, in higher plants both cytochrome b_6f complex and ATP synthase accumulation is strongly repressed within a few days (Kohzuma *et al.*, 2009). However, at least under controlled, non-stressed conditions in the greenhouse, the contents of both complexes change much more slowly over weeks, for example during leaf ontogenesis in tobacco (Schöttler *et al.*, 2004). This slow loss of the cytochrome b_6f complex and ATP synthase in mature leaves could be due to damage to the complex, resulting in their destabilization and ultimate degradation. Alternatively, the complexes may be actively degraded, to rebalance photosynthetic electron transport to the decreasing metabolic ATP and NADPH demand of ageing tobacco leaves (Schöttler *et al.*, 2007). However, so far, no proteases involved in cytochrome b_6f complex degradation have been identified in higher plants.

In *C. reinhardtii*, a rapid and specific degradation of the cytochrome b_6f complex can be induced under mixotrophic growth conditions and a low actinic light intensity when the nitrogen source is removed. Under photoautotrophic conditions, no such degradation of the cytochrome b_6f complex occurs. Nitrogen starvation under mixotrophic conditions results in a rapid arrest of growth. Photosynthetic electron transport is massively repressed within 24 h by the specific degradation of Rubisco, the cytochrome b_6f complex, and auxiliary proteins, which are involved in cytochrome b_6f complex biogenesis, while accumulation of the photosystems and ATP synthase remains unaltered (Wei *et al.*, 2014). The loss of the cytochrome b_6f complex and of some of the auxiliary proteins involved in its biogenesis, such as MCA1 and the CCB proteins (see 'Biogenesis of the cytochrome b_6f complex - Subunit synthesis and cofactor attachment') is much slower in mutants deficient in either the stromal Clp protease or the thylakoid-associated FtsH protease, which mainly degrades stroma-exposed sections of membrane-intrinsic thylakoid proteins. Therefore, both proteases have been implicated in the rapid degradation of the cytochrome b_6f complex and its auxiliary proteins (Majeran *et al.*, 2000; Wei *et al.*, 2014). A role for the FtsH protease in cytochrome b_6f complex degradation is also supported by the stabilization of cytochrome b_6f complex mutants affected in the binding of haem c_i to cytochrome b_6 in an FtsH mutant background (Malnoe *et al.*, 2014). Cytochrome b_6f complex degradation during nitrogen starvation is dependent on the presence of NO. NO could act as a signalling molecule inducing adaptor proteins of the proteases, which would recruit cytochrome b_6f complex subunits and auxiliary proteins for proteolysis. The presence of NO could also result in the post-translational modification of cytochrome b_6f complex subunits via nitrosylation, which

may prime them for proteolysis (Wei *et al.*, 2014). Also in response to a sulphur limitation, a rapid degradation of the cytochrome b_6f complex, together with PSII, occurs in darkness, and, again, this is dependent on the function of the Clp protease and the FtsH protease (Malnoe *et al.*, 2014).

The analysis of Clp protease mutants of *A. thaliana* has not provided indications for a similar involvement of this protease in cytochrome b_6f complex degradation in higher plants; rather, so far identified substrates suggest a housekeeping function of the Clp protease in the chloroplast stroma (Stanne *et al.*, 2009; Zybailov *et al.*, 2009). It remains to be determined if the imposition of a nutrient limitation on higher plants, which is similar to that triggering rapid degradation of the cytochrome b_6f complex in *C. reinhardtii*, could also result in a rapid degradation of the cytochrome b_6f complex. If so, that might open up the possibility to identify putative proteases involved in its degradation in higher plants.

The relatively rapid repression of ATP synthase contents in response to drought stress in wild watermelon (Kohzuma *et al.*, 2009; see 'Long-term responses of the photosynthetic apparatus during leaf ontogenesis and environmental acclimation') has been investigated in more detail, and it has been suggested that N-terminal acetylation of its ε-subunit protects ATP synthase from drought-induced degradation. The non-acetylated form of the ε-subunit seems to be rapidly degraded by an as yet unidentified chloroplast metalloaminopeptidase, which is induced during drought stress, so that the acetylation level of the ε-subunit determines ATP synthase stability (Hoshiyasu *et al.*, 2013). However, because the acetylase also remains to be identified, the precise regulation of ATP synthase stability and turnover during drought stress is still unknown.

Perspectives

Several decades of research have resulted in a detailed understanding of the adaptive responses of the photosynthetic apparatus in response to changing environmental conditions and metabolic and developmental states of the plants. Because the contribution of almost all components of the photosynthetic apparatus to flux control has been assessed using specific inhibitors and transgenic approaches during the last two decades (reviewed by Schöttler and Toth, 2014), many of the adaptive changes in complex accumulation can now be rationalized in terms of their contribution to flux control and the regulation of photosynthesis. However, we still do not know precisely how these adjustments of photosynthetic complex stoichiometry are achieved on a mechanistic level.

While some good indications exist for the signals involved in adjusting photosynthetic complex biogenesis and turnover, the actual signal transduction chains triggering changes in transcription and translation remain unknown. Our knowledge of the actual rate-limiting steps of complex accumulation is scarce, especially in the case of the cytochrome b_6f complex and ATP synthase, whose precise assembly pathways are still unknown. It also appears likely that for both ATP synthase and cytochrome b_6f complex biogenesis, several additional auxiliary proteins still remain to be identified, which possibly could contribute to the control of complex accumulation. For example, in the case of the cytochrome b_6f complex, it is still completely unknown how the 2Fe2S cluster is inserted into the Rieske apoprotein, and the existence of additional auxiliary proteins involved in this reaction can be predicted. The penultimate proof that complex assembly is fully understood, and that rate-limiting steps of complex biogenesis have been correctly identified, would be the successful overexpression of an entire complex by the specific up-regulation of the limiting subunit, or possibly the limiting auxiliary protein. This would also be of major importance for targeted modifications of the photosynthetic apparatus in synthetic biology.

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